

FORM PTO-1390 (REV. 11-2000)		U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE	ATTORNEY'S DOCKET NUMBER 11137/05006
TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371			U.S. APPLICATION NO. (If known, see 37 CFR 1.5) <b>09/936190</b>
INTERNATIONAL APPLICATION NO. PCT/US00/05915	INTERNATIONAL FILING DATE 8 March 2000 (08.03.00)	PRIORITY DATE CLAIMED 8 March 1999 (08.03.99)	
TITLE OF INVENTION GENETIC MANIPULATION OF ISOFLAVONOIDS			
APPLICANT(S) FOR DO/EO/US RICHARD A. DIXON, et al.			
Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:			
<p>1. <input checked="" type="checkbox"/> This is a <b>FIRST</b> submission of items concerning a filing under 35 U.S.C. 371.</p> <p>2. <input type="checkbox"/> This is a <b>SECOND</b> or <b>SUBSEQUENT</b> submission of items concerning a filing under 35 U.S.C. 371.</p> <p>3. <input type="checkbox"/> This is an express request to begin national examination procedures (35 U.S.C. 371(f)). The submission must include items (5), (6), (9) and (21) indicated below.</p> <p>4. <input type="checkbox"/> The US has been elected by the expiration of 19 months from the priority date (Article 31).</p> <p>5. <input checked="" type="checkbox"/> A copy of the International Application as filed (35 U.S.C. 371(c)(2))</p> <p style="margin-left: 20px;">a. <input type="checkbox"/> is attached hereto (required only if not communicated by the International Bureau).</p> <p style="margin-left: 20px;">b. <input type="checkbox"/> has been communicated by the International Bureau.</p> <p style="margin-left: 20px;">c. <input checked="" type="checkbox"/> is not required, as the application was filed in the United States Receiving Office (RO/US).</p> <p>6. <input type="checkbox"/> An English language translation of the International Application as filed (35 U.S.C. 371(c)(2)).</p> <p style="margin-left: 20px;">a. <input type="checkbox"/> is attached hereto.</p> <p style="margin-left: 20px;">b. <input type="checkbox"/> has been previously submitted under 35 U.S.C. 154(d)(4).</p> <p>7. <input checked="" type="checkbox"/> Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3))</p> <p style="margin-left: 20px;">a. <input type="checkbox"/> are attached hereto (required only if not communicated by the International Bureau).</p> <p style="margin-left: 20px;">b. <input type="checkbox"/> have been communicated by the International Bureau.</p> <p style="margin-left: 20px;">c. <input type="checkbox"/> have not been made; however, the time limit for making such amendments has NOT expired.</p> <p style="margin-left: 20px;">d. <input checked="" type="checkbox"/> have not been made and will not be made.</p> <p>8. <input type="checkbox"/> An English language translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).</p> <p>9. <input type="checkbox"/> An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)).</p> <p>10. <input type="checkbox"/> An English language translation of the annexes of the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).</p> <p><b>Items 11 to 20 below concern document(s) or information included:</b></p> <p>11. <input type="checkbox"/> An Information Disclosure Statement under 37 CFR 1.97 and 1.98.</p> <p>12. <input type="checkbox"/> An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.</p> <p>13. <input checked="" type="checkbox"/> A FIRST preliminary amendment.</p> <p>14. <input type="checkbox"/> A SECOND or SUBSEQUENT preliminary amendment.</p> <p>15. <input type="checkbox"/> A substitute specification.</p> <p>16. <input type="checkbox"/> A change of power of attorney and/or address letter.</p> <p>17. <input checked="" type="checkbox"/> A computer-readable form of the sequence listing in accordance with PCT Rule 13ter.2 and 35 U.S.C. 1.821 - 1.825.</p> <p>18. <input type="checkbox"/> A second copy of the published international application under 35 U.S.C. 154(d)(4).</p> <p>19. <input type="checkbox"/> A second copy of the English language translation of the international application under 35 U.S.C. 154(d)(4).</p> <p>20. <input checked="" type="checkbox"/> Other items or information: Statement Under WIPO Standard ST.25</p>			
Express Mail No.: EL 794556213 US		10 September 2001	
I hereby certify that this application is being deposited with the United States Post Office to Addressee" service under 37 CFR 1.10 on the date indicated above			

page 1 of 2 and is addressed to the Assistant Commissioner for Patents, Box PCT, Washington, D.C. 20231.

Lottie Davis

(Typed or Printed Name of Person Mailing Application )

*Lottie Davis*  
(Signature of Person)

U.S. APPLICATION NO. (required) <b>09/936190</b>		INTERNATIONAL APPLICATION NO. <b>PCT/US00/05915</b>		ATTORNEY'S DOCKET NUMBER <b>11137/05006</b>	
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21. <input checked="" type="checkbox"/> The following fees are submitted: <b>BASIC NATIONAL FEE (37 CFR 1.492 (a) (1) - (5)):</b> Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO and International Search Report not prepared by the EPO or JPO ..... <b>\$1000.00</b>  International preliminary examination fee (37 CFR 1.482) not paid to USPTO but International Search Report prepared by the EPO or JPO ..... <b>\$860.00</b>  International preliminary examination fee (37 CFR 1.482) not paid to USPTO but international search fee (37 CFR 1.445(a)(2)) paid to USPTO ..... <b>\$710.00</b>  International preliminary examination fee (37 CFR 1.482) paid to USPTO but all claims did not satisfy provisions of PCT Article 33(1)-(4) ..... <b>\$690.00</b>  International preliminary examination fee (37 CFR 1.482) paid to USPTO and all claims satisfied provisions of PCT Article 33(1)-(4) ..... <b>\$100.00</b>  <b>ENTER APPROPRIATE BASIC FEE AMOUNT =</b>				<b>CALCULATIONS PTO USE ONLY</b>	
				\$ 860.00	
Surcharge of <b>\$130.00</b> for furnishing the oath or declaration later than <input type="checkbox"/> 20 <input checked="" type="checkbox"/> 30 months from the earliest claimed priority date (37 CFR 1.492(e)).				\$ 130.00	
CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE	\$	
Total claims	92 - 20 =	72	x \$18.00	\$1,296.00	
Independent claims	21 - 3 =	18	x \$80.00	\$1,440.00	
MULTIPLE DEPENDENT CLAIM(S) (if applicable)				+ \$270.00	
<b>TOTAL OF ABOVE CALCULATIONS =</b>				<b>\$3,996.00</b>	
<input checked="" type="checkbox"/> Applicant claims small entity status. See 37 CFR 1.27. The fees indicated above are reduced by 1/2.				+ \$1,998.00	
<b>SUBTOTAL =</b>				<b>\$1,998.00</b>	
Processing fee of <b>\$130.00</b> for furnishing the English translation later than <input type="checkbox"/> 20 <input type="checkbox"/> 30 months from the earliest claimed priority date (37 CFR 1.492(f)).				\$	
<b>TOTAL NATIONAL FEE =</b>				<b>\$1,998.00</b>	
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). <b>\$40.00</b> per property +				\$	
<b>TOTAL FEES ENCLOSED =</b>				<b>\$1,998.00</b>	
				Amount to be refunded:	\$
				charged:	\$

a. ☐ A check in the amount of \$ \_\_\_\_\_ to cover the above fees is enclosed.

b. ☒ Please charge my Deposit Account No. 18-1260 in the amount of \$ 1,998.00 to cover the above fees.  
 A duplicate copy of this sheet is enclosed.

c. ☒ The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any  
 overpayment to Deposit Account No. 18-1260. A duplicate copy of this sheet is enclosed.

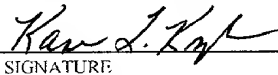
d. ☐ Fees are to be charged to a credit card. **WARNING:** Information on this form may become public. Credit card  
 information should not be included on this form. Provide credit card information and authorization on PTO-2038.

**NOTE:** Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR  
 1.137 (a) or (b)) must be filed and granted to restore the application to pending status.

SEND ALL CORRESPONDENCE TO.

Eugenia S. Hansen  
 Sidley Austin Brown & Wood  
 717 N. Harwood, Suite 3400  
 Dallas, Texas 75201

  
 SIGNATURE  
 Karen L. Knezek  
 NAME  
 39,253  
 REGISTRATION NUMBER

Applicant or Patentee: **Richard A. Dixon, et al.**

Serial or Patent No.: **Unassigned**

Filed or Issued:

Attorney Docket No.: **11137/05006**

For: **GENETIC MANIPULATION OF ISOFLAVONOIDS**

**VERIFIED STATEMENT (DECLARATION)  
CLAIMING SMALL ENTITY STATUS  
(37 C.F.R. §§ 1.9(f) and 1.27(d))  
NONPROFIT ORGANIZATION**

I hereby declare that I am an official empowered to act on behalf of the nonprofit organization identified below:

NAME OF ORGANIZATION: **The Samuel Roberts Noble Foundation, Inc.**

ADDRESS OF ORGANIZATION: 2510 Sam Noble Parkway, Ardmore, Oklahoma 73402

TYPE OF ORGANIZATION

- ☐ UNIVERSITY OR OTHER INSTITUTE OF HIGHER EDUCATION
- ☒ TAX EXEMPT UNDER INTERNAL REVENUE SERVICE CODE (26 U.S.C. §§ 501(a) and 501(c)(3))
- ☐ NONPROFIT SCIENTIFIC OR EDUCATIONAL UNDER STATUTE OF STATE OF THE UNITED STATES OF AMERICA  
(NAME OF STATE )  
(CITATION OF STATUTE )
- ☐ WOULD QUALIFY AS TAX EXEMPT UNDER INTERNAL REVENUE SERVICE CODE (26 U.S.C. §§ 501(a) AND 501(c)(3)) IF LOCATED IN THE UNITED STATES OF AMERICA
- ☐ WOULD QUALIFY AS NONPROFIT SCIENTIFIC OR EDUCATIONAL UNDER STATUTE OF STATE OF THE UNITED STATES OF AMERICA IF LOCATED IN THE UNITED STATES OF AMERICA  
(NAME OF STATE )  
(CITATION OF STATUTE )

I hereby declare that the nonprofit organization identified above qualifies as a nonprofit organization as defined in 37 C.F.R. § 1.9(e) for purposes of paying reduced fees under §§ 41(a) and (b) of Title 35, United States Code, with regard to the invention entitled **GENETIC MANIPULATION OF ISOFLAVONOIDs** by inventors **Richard A. Dixon and Christopher L. Steele** described in

- ☐ the specification filed herewith.
- ☒ PCT International Application No. PCT/US00/05915, filed March 8, 2000.
- ☐ patent no. , issued .

I hereby declare that rights under contract or law have been conveyed to and remain with the nonprofit organization regarding the above-identified invention.

If the rights held by the nonprofit organization are not exclusive, each individual, concern or organization having rights in the invention is listed below\* and that no rights to the invention are held by any person, other than the inventor, who would not qualify as an independent inventor under 37 C.F.R. § 1.9(c) if that person made the invention, or by any concern which would not qualify as a small business concern under 37 C.F.R. § 1.9(d) or a nonprofit organization under 37 C.F.R. § 1.9(e).

Each person, concern or organization having any rights in the invention is listed below:

- ☒ no such person, concern or organization
- ☐ persons, concerns or organizations listed below\*

\*NOTE: Separate verified statements are required from each named person, concern, or organization having rights to the invention averring to their status as small entities. (37 C.F.R. § 1.27)

NAME: N/A

ADDRESS:

☐ individual      ☐ small business concern      ☐ nonprofit organization

I acknowledge the duty to file, in this application or patent, notification of any change in status resulting in loss of entitlement to small entity status prior to paying, or at the time of paying, the earliest of the issue fee or any maintenance fee due after the date on which status as a small entity is no longer appropriate. (37 C.F.R. § 1.28(b))

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are

punishable by fine or imprisonment, or both, under § 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application, any patent issuing thereon, or any patent to which this verified statement is directed.

NAME OF PERSON SIGNING: **Michael A. Cawley**

TITLE IN ORGANIZATION: **President and CEO**

ADDRESS OF PERSON SIGNING: **2510 Sam Noble Parkway, Ardmore OK 73402**

SIGNATURE Michael A. Cawley

DATE 9.4.01

## IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of:

Richard A. Dixon, et al.


For: **GENETIC MANIPULATION OF ISOFLAVONOIDs**

Application Serial No. Unassigned

Filing Date: Concurrently herewith

International  
Application No.: PCT/US00/05915International  
Filing Date: 08 March 2000Assistant Commissioner for Patents  
Box PCT  
Washington, D.C. 20231

EXPRESS MAIL NO. EL794556213US DATE OF DEPOSIT 10 September 2001  
I hereby certify that this correspondence is being deposited with the United States  
Postal Service "Express Mail Post Office to Addressee" service under 37 CFR 1.10  
on the date indicated above and is addressed to the Assistant Commissioner for  
Patents, Box PCT, Washington, D.C. 20231 on 10 September 2001  
(Date of Deposit)

Lottie Davis  
Name of Depositor

Signature

Date of Signature: 10 September 2001

Dear Sir:

**PRELIMINARY AMENDMENT**

This preliminary amendment is being filed concurrently with the 35 U.S.C. 371 national stage filing of International Application No. PCT/US00/05915. In accordance with 37 C.F.R. §1.121(c)(3), this document implements changes to the claims by presenting an entire set of pending claims. An Appendix entitled Version With Markings to Show Changes Made, is attached showing the current amendments to the specification and/or claims in marked form. Any claim not accompanied by a marked up version should be construed as not having been changed relative to the immediate prior version thereof, if any.

**IN THE CLAIMS**

Please replace the previous version of the claims with the following clean version, wherein all pending Claims 1-65 are deleted and new Claims 1-69 have been added.

1. A method for introducing into a naturally non-isoflavonoid-producing plant species the enzyme catalyzing the aryl migration of a flavanone to form an isoflavanone intermediate or an isoflavone, comprising:

introducing a DNA segment encoding said enzyme into said plant to form a transgenic plant, wherein said transgenic plant expresses said DNA segment under the control of a suitable constitutive or inducible promoter when said transgenic plant is exposed to conditions which permit expression.

2. The method of Claim 1, wherein chalcone synthase, chalcone reductase, and chalcone isomerase genes are also expressed in said plant to cause in vivo formation of daidzein or a daidzein derivative.

3. The method of Claim 2, wherein said plant is further transformed to comprise said chalcone synthase, chalcone reductase, and chalcone isomerase genes.

4. The method of Claim 1 or 2, wherein said plant further comprises downstream genes to metabolize said formed isoflavanone intermediate or isoflavone to biologically active isoflavonoid derivatives or conjugates.

5. The method of Claim 4, wherein said downstream gene is selected from the group consisting of isoflavone *O*-methyltransferase, isoflavone 2'-hydroxylase, isoflavone reductase, and vestitone reductase.

6. The method of Claim 5, wherein said plant comprises down-stream gene 4'-*O*-methyltransferase to form biochanin A or a biochanin A derivative.

7. A method for increasing the level of isoflavonoid compounds in naturally isoflavonoid-producing plants comprising:

introducing a DNA segment encoding the enzyme catalyzing the aryl migration of a flavanone to yield an isoflavonoid to form a transgenic plant, wherein said transgenic plant expresses said DNA segment under the control of a suitable constitutive or inducible promoter when said transgenic plant is exposed to conditions which permit expression.

8. The method of Claim 7, wherein said isoflavonoid is selected from the group consisting of an isoflavonone intermediate, an isoflavone, an isoflavone derivative, and an isoflavone conjugate.

9. The method of Claim 1 or 7, wherein said DNA segment comprises isolated genomic DNA.

10. The method of Claim 1 or 7, wherein said DNA segment comprises recombinant cDNA.

11. The method of Claim 1 or 7, wherein said DNA segment comprises CYP93C gene.

12. The method of Claim 11, wherein said gene consists of the sequence from nucleotide 36 to nucleotide 1598 of the sequence depicted in SEQ ID NO:1.

13. The method of Claim 1 or 7, wherein said DNA segment is a *Medicago truncatula* homolog of a CYP93C gene.

14. The method of Claim 12, wherein said gene consists of the sequence from nucleotide 92 to nucleotide 1657 of the sequence depicted in SEQ ID NO:4.

15. The method of Claim 1 or 7, wherein said flavanone is liquiritigenin.

16. The method of Claim 1 or 7, wherein said flavanone is naringenin.

17. The method of Claim 1 or 7, wherein said transgenic plant possesses an isoflavonoid which is isolated from said plant and used to prepare a food.

18. The method of Claim 1 or 7, wherein said transgenic plant possesses an isoflavonoid which is isolated from said plant and used to prepare a food stuff, a nutritional supplement, an animal feed supplement, a nutraceutical, or a pharmaceutical.

19. The method of Claim 1 or 7, wherein said transgenic plant possesses an isoflavonoid which provides a pharmaceutical benefit to a patient.

20. A method for synthesizing an isoflavonone intermediate or an isoflavone from a flavanone by expressing a recombinant CYP93C gene segment in a suitable bacterial, fungal, algal, or insect cell system.



21. A method of reducing the levels of isoflavonoid compounds in a naturally isoflavonoid-producing plant comprising introducing and expressing an antisense or gene silencing construct that contains an intact CYP93C gene or segments thereof into said plant.

22. The method of Claim 20 or 21, wherein said gene consists of the sequence from nucleotide 36 to nucleotide 1598 of the sequence depicted in SEQ ID NO:1.

23. The method of Claim 20 or 21, wherein said gene consists of the sequence from nucleotide 92 to nucleotide 1657 of the sequence depicted in SEQ ID NO:4.

24. A naturally non-isoflavonoid producing plant cell transformed by introducing a DNA segment encoding the enzyme catalyzing the aryl migration of a flavanone to form an isoflavanone intermediate or an isoflavone, wherein said transgenic plant cell expresses said DNA segment under the control of a suitable constitutive or inducible promoter when exposed to conditions which permit expression.

25. The plant cell of Claim 24, wherein chalcone synthase, chalcone reductase, and chalcone isomerase genes are also expressed in said plant to cause in vivo formation of daidzein or a daidzein derivative.

26. The plant cell of Claim 25, wherein said plant cell is further transformed to comprise said chalcone synthase, chalcone reductase, and chalcone isomerase genes.

27. The plant cell of Claim 24 or 25, wherein said plant cell further comprises downstream genes to metabolize said formed isoflavanone intermediate or isoflavone to biologically active isoflavonoid derivatives or conjugates.

28. The plant cell of Claim 27, wherein said downstream gene is selected from the group consisting of isoflavone *O*-methyltransferase, isoflavone 2'-hydroxylase, isoflavone reductase, and vestitone reductase.

29. The plant cell of Claim 28, wherein said plant cell comprises downstream gene 4'-*O*-methyltransferase to form biochanin A or a biochanin A derivative.

30. A naturally isoflavonoid-producing plant cell transformed by introducing a DNA segment encoding the enzyme catalyzing the aryl migration of a flavanone to yield an isoflavonoid to form a transformed plant cell, wherein said transformed plant cell expresses said DNA segment under the control of a suitable constitutive or inducible promoter when exposed to conditions which permit expression.

31. The plant cell of Claim 30, wherein said isoflavonoid is selected from the group consisting of an isoflavonone intermediate, an isoflavone, an isoflavone derivative, and an isoflavone conjugate.

32. The plant cell of Claim 24, 30 or 31, wherein said DNA segment comprises isolated genomic DNA.

33. The plant cell of Claim 24, 30 or 31, wherein said DNA segment comprises recombinant cDNA.

34. The plant cell of Claim 24, 30 or 31, wherein said DNA segment comprises CYP93C gene.

35. The plant cell of Claim 34, wherein said DNA segment consists of the sequence from nucleotide 36 to nucleotide 1598 of the sequence depicted in SEQ ID NO:1.

36. The plant cell of Claim 24, 30 or 31, wherein said DNA segment is a *Medicago truncatula* homolog of a CYP93C gene.

37. The plant cell of Claim 36, wherein said gene consists of the sequence from nucleotide 92 to nucleotide 1657 of the sequence depicted in SEQ ID NO:4.

38. A transgenic plant cell having reduced levels of isoflavonoid compounds, said plant cell transformed by introducing an antisense or gene silencing construct that contains an intact CYP93C gene or segments thereof into said plant cell.

39. The plant cell of Claim 38, wherein said gene consists of the sequence from nucleotide 36 to nucleotide 1598 of the sequence depicted in SEQ ID NO:1.

40. The plant cell of Claim 38, wherein said gene consists of the sequence from nucleotide 92 to nucleotide 1657 of the sequence depicted in SEQ ID NO:4.

41. An isolated gene or DNA segment comprising a portion which encodes a cytochrome P450 of the CYP93 family that can catalyze the aryl migration of a flavanone to yield an isoflavanone intermediate or an isoflavone, wherein said portion consists of nucleotide 36 to nucleotide 1598 of the sequence depicted in SEQ ID NO:1.

42. The gene or DNA segment of Claim 41, wherein said gene is the soybean gene encoding the enzyme catalyzing the aryl migration of liquiritigenin.

43. The gene or DNA segment of Claim 41, wherein said gene is the soybean gene encoding the enzyme catalyzing the aryl migration of naringenin.

44. A protein encoded by a portion of an isolated gene or DNA segment which encodes a cytochrome P450 that can catalyze the aryl migration of a flavanone to yield an isoflavanone intermediate or an isoflavone, wherein said portion consists of nucleotide 36 to nucleotide 1598 of the sequence depicted in SEQ ID NO:1.

45. An isolated gene or DNA segment comprising a portion which encodes a cytochrome P450 that can catalyze the aryl migration of a flavanone to yield an isoflavanone intermediate or an isoflavone, wherein said portion is a *Medicago truncatula* homolog of a CYP93C gene.

46. The gene or DNA segment of Claim 45 consisting of nucleotide 92 to nucleotide 1657 of the sequence depicted in SEQ ID NO:4.

47. The gene or DNA segment of Claims 45 or 46, wherein said gene is the *Medicago truncatula* gene encoding the enzyme catalyzing the aryl migration of liquiritigenin.

48. The gene or DNA segment of Claims 45 or 46, wherein said gene is the *Medicago truncatula* gene encoding the enzyme catalyzing the aryl migration of naringenin.

49. A protein encoded by a portion of an isolated gene or DNA segment which encodes a cytochrome P450 that can catalyze the aryl migration of a flavanone to yield an isoflavanone intermediate or an isoflavone, wherein said portion is a *Medicago truncatula* homolog of a CYP93C gene.

50. A transgenic plant transformed with an isolated gene or DNA segment which encodes a cytochrome P450 that can catalyze the aryl migration of a flavanone to yield an isoflavanone intermediate or an isoflavone, wherein said transgenic plant exhibits increased levels of an isoflavonoid when compared to the level of said isoflavonoid in plants of the same species which do not comprise said isolated gene or DNA segment.

51. The transgenic plant of Claim 50, wherein the level of bacterial or fungal symbiosis is increased.

52. The transgenic plant of Claim 50, wherein at least a portion of said transgenic plant is made into a composition suitable for ingestion as a food stuff, a nutritional supplement, an animal feed supplement, or a nutraceutical.

53. The transgenic plant of Claim 50, wherein at least a portion of said edible transgenic plant material capable of being ingested for its nutritional value is made into a food.

54. A method of preparing a nutraceutical composition for achieving a nutritional effect using a transgenic plant transformed with an isolated gene or DNA segment which encodes a cytochrome P450 that can catalyze the aryl migration of a flavanone to yield an isoflavanone intermediate or an isoflavone, wherein said transgenic plant exhibits increased levels of an isoflavonoid when compared to the level of said isoflavonoid in plants of the same species which do not comprise said isolated gene or DNA segment.

55. A method of preparing a pharmaceutical composition for achieving a therapeutic effect using a transgenic plant transformed with an isolated gene or DNA segment which encodes a cytochrome P450 that can catalyze the aryl migration of a flavanone to yield an isoflavanone intermediate or an isoflavone, wherein said transgenic plant exhibits increased levels of an isoflavonoid when compared to the level of said isoflavonoid in plants of the same species which do not comprise said isolated gene or DNA segment.

56. A method of using a transgenic plant transformed with an isolated gene or DNA segment which encodes a cytochrome P450 that can catalyze the aryl migration of a flavanone to yield an isoflavanone intermediate or an isoflavone, wherein said transgenic plant exhibits increased levels of an isoflavonoid when compared to the level of said isoflavonoid in plants of the same species which do not comprise said isolated gene or DNA segment to provide a nutraceutical benefit to a human or animal administered said isoflavonoid.

57. The method of Claim 56, wherein said isoflavonoid is administered by ingestion of at least a portion of said plant.

58. The method of Claim 56, wherein said isoflavonoid is administered by ingestion of a composition comprising an isoflavonoid isolated from said plant.

59. A method of transforming a plant with an isolated gene or DNA segment which encodes a cytochrome P450 that can catalyze the aryl migration of a flavanone to yield an isoflavanone intermediate or an isoflavone, wherein said transgenic plant exhibits increased levels of an isoflavonoid when compared to the level of said isoflavonoid in plants of the same species which do not comprise said isolated gene or DNA segment.

60. The method of Claim 59, wherein the nutritional value of said plant is increased.

61. The method of Claim 59, wherein the disease resistance in said plant is increased.

62. The method of Claim 59, wherein bacterial or fungal symbiosis in said plant is increased.

63. The method of claim 59, wherein said plant is a leguminous plant.

64. The method of claim 63, wherein the nodulation efficiency of said plant is increased.

65. A leguminous transgenic plant exhibiting increased nodulation efficiency, wherein said transgenic plant is transformed with an isolated gene or DNA segment which encodes a cytochrome P450 that can catalyze the aryl migration of a flavanone to yield an isoflavanone intermediate or an isoflavone, wherein said transgenic plant exhibits increased levels of an isoflavonoid when compared to the level of said isoflavonoid in plants of the same species which do not comprise said isolated gene or DNA segment.

66. A transgenic plant comprising at least one recombinant DNA sequence encoding a cytochrome P450 that can catalyze the aryl migration of a flavanone to yield an isoflavanone intermediate or an isoflavone, wherein said transgenic plant exhibits an increased level of an isoflavonoid when compared to the level of said isoflavonoid in plants of the same species which do not comprise said recombinant DNA sequence.

67. Seed from a transgenic plant comprising at least one recombinant DNA sequence encoding a cytochrome P450 that can catalyze the aryl migration of a flavanone to yield an isoflavanone intermediate or an isoflavone, wherein said transgenic plant exhibits an increased level of an isoflavonoid when compared to the level of said isoflavonoid in plants of the same species which do not comprise said recombinant DNA sequence.

68. Progeny from a transgenic plant comprising at least one recombinant DNA sequence encoding a cytochrome P450 that can catalyze the aryl migration of a flavanone to yield an isoflavanone intermediate or an isoflavone, wherein said transgenic plant exhibits an increased level of an isoflavonoid when compared to the level of said isoflavonoid in plants of the same species which do not comprise said recombinant DNA sequence.


69. Progeny from seed of a transgenic plant comprising at least one recombinant DNA sequence encoding a cytochrome P450 that can catalyze the aryl migration of a flavanone to yield an isoflavanone intermediate or an isoflavone, wherein said transgenic plant exhibits an increased level of an isoflavonoid when compared to the level of said isoflavonoid in plants of the same species which do not comprise said recombinant DNA sequence.

**REMARKS**

Prior to this Preliminary Amendment, Claims 1-65 as presented in the annex to the International Preliminary Examination Report were pending. With this Preliminary Amendment, all pending Claims 1-65 are deleted and new Claims 1-69 are added. Payment for the fees associated with the addition of these claims has been directed on the Transmittal Letter to the United States Designated/Elected Office Concerning a Filing Under 35 U.S.C. §371 filed concurrently herewith.

Respectfully submitted,

By:

  
Karen L. Knezek  
Registration No. 39,253

KLK:ld

10 September 2001

SIDLEY AUSTIN BROWN & WOOD

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(214) 981-3300

(214) 981-3400 (FAX)

**APPENDIX: VERSION WITH MARKINGS TO SHOW CHANGES MADE**

[1. A method for introducing into a naturally non-isoflavonoid-producing plant species the enzyme catalyzing the aryl migration of a flavanone to form an isoflavanone intermediate or an isoflavone, comprising:

introducing a DNA segment encoding said enzyme into said plant to form a transgenic plant,

5 wherein said transgenic plant expresses said DNA segment under the control of a suitable constitutive or inducible promoter when said transgenic plant is exposed to conditions which permit expression.]

[2. The method of Claim 1, wherein chalcone synthase, chalcone reductase, and chalcone isomerase genes are also expressed in said plant to cause in vivo formation of daidzein or a daidzein derivative.]

[3. The method of Claim 2, wherein said plant is further transformed to comprise said chalcone synthase, chalcone reductase, and chalcone isomerase genes.]

[4. The method of Claim 1 or 2, wherein said plant further comprises downstream genes to metabolize said formed isoflavanone intermediate or isoflavone to biologically active isoflavonoid derivatives or conjugates.]

[5. The method of Claim 4, wherein said downstream gene is selected from the group consisting of isoflavone *O*-methyltransferase, isoflavone 2'-hydroxylase, isoflavone reductase, and vestitone reductase.]

[6. The method of Claim 5, wherein said plant comprises downstream gene 4'-*O*-methyltransferase to form biochanin A or a biochanin A derivative.]

[7. A method for increasing the level of isoflavonoid compounds in naturally isoflavonoid-producing plants comprising:

introducing a DNA segment encoding the enzyme catalyzing the aryl migration of a flavanone to yield an isoflavonoid to form a transgenic plant, wherein said transgenic plant expresses said

5 DNA segment under the control of a suitable constitutive or inducible promoter when said transgenic plant is exposed to conditions which permit expression.]



[8. The method of Claim 7, wherein said isoflavonoid is selected from the group consisting of an isoflavonone intermediate, an isoflavone, an isoflavone derivative, and an isoflavone conjugate.]

[9. The method of Claim 1, 7 or 8, wherein said DNA segment comprises isolated genomic DNA.]

[10. The method of Claim 1, 7 or 8, wherein said DNA segment comprises recombinant cDNA.]

[11. The method of Claim 7-10, wherein said DNA segment comprises CYP93C gene.]

[12. The method of Claim 7-10, wherein said DNA segment is a *Medicago truncatula* homolog of a CYP93C gene.]

[13. The method of Claim 1-12, wherein said flavanone is liquiritigenin.]

[14. The method of Claim 1-12, wherein said flavanone is naringenin.]

[15. A method for synthesizing an isoflavanone intermediate or an isoflavone from a flavanone by expressing a recombinant CYP93C gene segment in a suitable bacterial, fungal, algal, or insect cell system.]

[16. A method of reducing the levels of isoflavonoid compounds in a naturally isoflavonoid-producing plant comprising introducing and expressing an antisense or gene silencing construct that contains an intact CYP93C gene or segments thereof into said plant.]

[17. The method of Claim 1, 11, 15 or 16, wherein said gene consists of the sequence from nucleotide 36 to nucleotide 1598 of the sequence depicted in SEQ ID NO:1.]

[18. The method of Claim 1, 12, 15 or 16, wherein said gene consists of the sequence from nucleotide 92 to nucleotide 1657 of the sequence depicted in SEQ ID NO:4.]

[19. A naturally non-isoflavonoid producing plant cell transformed by introducing a DNA segment encoding the enzyme catalyzing the aryl migration of a flavanone to form an isoflavanone intermediate or an isoflavone, wherein said transgenic plant cell expresses said DNA segment under the control of a suitable constitutive or inducible promoter when exposed to conditions which permit expression.]

5

[20. The plant cell of Claim 19, wherein chalcone synthase, chalcone reductase, and chalcone isomerase genes are also expressed in said plant to cause in vivo formation of daidzein or a daidzein derivative.]

[21. The plant cell of Claim 20, wherein said plant cell is further transformed to comprise said chalcone synthase, chalcone reductase, and chalcone isomerase genes.]

[22. The plant cell of Claim 19-20, wherein said plant cell further comprises downstream genes to metabolize said formed isoflavanone intermediate or isoflavone to biologically active isoflavonoid derivatives or conjugates.]

[23. The plant cell of Claim 22, wherein said downstream gene is selected from the group consisting of isoflavone *O*-methyltransferase, isoflavone 2'-hydroxylase, isoflavone reductase, and vestitone reductase.]

[24. The plant cell of Claim 23, wherein said plant cell comprises downstream gene 4'-*O*-methyltransferase to form biochanin A or a biochanin A derivative.]

[25. A naturally isoflavonoid-producing plant cell transformed by introducing a DNA segment encoding the enzyme catalyzing the aryl migration of a flavanone to yield an isoflavonoid to form a transformed plant cell, wherein said transformed plant cell expresses said DNA segment under the control of a suitable constitutive or inducible promoter when exposed to  
5 conditions which permit expression.]

[26. The plant cell of Claim 25, wherein said isoflavonoid is selected from the group consisting of an isoflavanone intermediate, an isoflavone, an isoflavone derivative, and an isoflavone conjugate.]

[27. The plant cell of Claim 19, 25 or 26, wherein said DNA segment comprises isolated genomic DNA.]

[28. The plant cell of Claim 19, 25 or 26, wherein said DNA segment comprises recombinant cDNA.]

[29. The plant cell of Claim 19 or 25-28, wherein said DNA segment comprises CYP93C gene.]

[30. The plant cell of Claim 19 or 25-28, wherein said DNA segment is a *Medicago truncatula* homolog of a CYP93C gene.]

[31. A transgenic plant cell having reduced levels of isoflavonoid compounds, said plant cell transformed by introducing an antisense or gene silencing construct that contains an intact CYP93C gene or segments thereof into said plant cell.]

[32. The plant cell of Claim 29 or 31, wherein said gene consists of the sequence from nucleotide 36 to nucleotide 1598 of the sequence depicted in SEQ ID NO:1.]

[33. The plant cell of Claim 30 or 31, wherein said gene consists of the sequence from nucleotide 92 to nucleotide 1657 of the sequence depicted in SEQ ID NO:4.]

[34. An isolated gene or DNA segment comprising a portion which encodes a cytochrome P450 of the CYP93 family that can catalyze the aryl migration of a flavanone to yield an isoflavanone intermediate or an isoflavone, wherein said portion consists of nucleotide 36 to nucleotide 1598 of the sequence depicted in SEQ ID NO:1.]

[35. The gene or DNA segment of Claim 34, wherein said gene is the soybean gene encoding the enzyme catalyzing the aryl migration of liquiritigenin.]

[36. The gene or DNA segment of Claim 34, wherein said gene is the soybean gene encoding the enzyme catalyzing the aryl migration of naringenin.]

[37. A protein encoded by a portion of an isolated gene or DNA segment which encodes a cytochrome P450 that can catalyze the aryl migration of a flavanone to yield an isoflavanone intermediate or an isoflavone, wherein said portion consists of nucleotide 36 to nucleotide 1598 of the sequence depicted in SEQ ID NO:1.]

[38. An isolated gene or DNA segment comprising a portion which encodes a cytochrome P450 that can catalyze the aryl migration of a flavanone to yield an isoflavanone intermediate or an isoflavone, wherein said portion is a *Medicago truncatula* homolog of a CYP93C gene.]

[39. The gene or DNA segment of Claim 38 consisting of nucleotide 92 to nucleotide 1657 of the sequence depicted in SEQ ID NO:4.]

[40. The gene or DNA segment of Claims 38 or 39, wherein said gene is the *Medicago truncatula* gene encoding the enzyme catalyzing the aryl migration of liquiritigenin.]

[41. The gene or DNA segment of Claims 38 or 39, wherein said gene is the *Medicago truncatula* gene encoding the enzyme catalyzing the aryl migration of naringenin.]

[42. A protein encoded by a portion of an isolated gene or DNA segment which encodes a cytochrome P450 that can catalyze the aryl migration of a flavanone to yield an isoflavanone intermediate or an isoflavone, wherein said portion is a *Medicago truncatula* homolog of a CYP93C gene.]

[43. A transgenic plant cell transformed with an isolated gene or DNA segment which encodes a cytochrome P450 that can catalyze the aryl migration of a flavanone to yield an isoflavanone intermediate or an isoflavone, wherein said transgenic plant cell exhibits increased levels of an isoflavonoid when compared to the level of said isoflavonoid in plant cells of the same species which do not comprise said isolated gene or DNA segment.]

[44. A food comprising edible transgenic plant material capable of being ingested for its nutritional value, wherein said transgenic plant comprises plant cells according to claim 43.]

[45. A method of preparing a food comprising at least one isoflavonoid comprising: transforming a plant according to the method of claim 1 or 7, wherein said transgenic plant exhibits increased levels of an isoflavonoid when compared to the level of said isoflavonoid in plants of the same species which do not comprise said DNA segment, isolating said isoflavonoid and incorporating into said food.]

[46. A composition comprising at least a portion of a transgenic plant according to claim 43, wherein said composition is suitable for ingestion as a food stuff, a nutritional supplement, an animal feed supplement, or a nutraceutical.]

[47. A method of preparing a composition comprising an isoflavonoid suitable for administration as a food stuff, a nutritional supplement, an animal feed supplement, a nutraceutical, or a pharmaceutical, comprising: transforming a plant according to the method of claim 1 or 7, wherein said transgenic plant exhibits increased levels of an isoflavonoid when compared to the level of said isoflavonoid in

plants of the same species which do not comprise said DNA segment, isolating said isoflavonoid and incorporating into said compositions.]

[48. A method of using a transgenic plant according to claim 43 to provide a nutraceutical benefit to a human or animal administered said isoflavonoid.]

[49. The method of Claim 48, wherein said isoflavonoid is administered by ingestion of at least a portion of said plant.]

[50. The method of Claim 48, wherein said isoflavonoid is administered by ingestion of a composition comprising an isoflavonoid isolated from said plant.]

[51. A method for making a pharmaceutical preparation, comprising:  
transforming a plant according to the method of claim 1 or 7, wherein said transgenic plant exhibits increased levels of an isoflavonoid when compared to the level of said isoflavonoid in plants of the same species which do not comprise said DNA segment, isolating said isoflavonoid and formulating said isoflavonoid to form a pharmaceutical preparation.]

[52. A method of transforming a plant with an isolated gene or DNA segment which encodes a cytochrome P450 that can catalyze the aryl migration of a flavanone to yield an isoflavanone intermediate or an isoflavone, wherein said transgenic plant exhibits increased levels of an isoflavonoid when compared to the level of said isoflavonoid in plants of the same species which do not comprise said isolated gene or DNA segment.]

[53. A method of Claim 52, wherein the nutritional value of said plant is increased.]

[54. A method of Claim 52, wherein the disease resistance in said plant is increased.]

[55. A method of Claim 52, wherein bacterial or fungal symbiosis in said plant is increased.]

[56. A method of claim 52, wherein said plant is a leguminous plant.]

[57. A method of claim 56, wherein the nodulation efficiency of said plant is increased.]

[58. A leguminous transgenic plant exhibiting increased nodulation efficiency, wherein said transgenic plant is transformed according to the method of Claim 52.]

[59. A transgenic plant of Claim 43 exhibiting an increased level of bacterial or fungal symbiosis.]

[60. A transgenic plant comprising at least one recombinant DNA sequence encoding a cytochrome P450 that can catalyze the aryl migration of a flavanone to yield an isoflavanone intermediate or an isoflavone, wherein said transgenic plant exhibits an increased level of an isoflavonoid when compared to the level of said isoflavonoid in plants of the same species which  
5 do not comprise said recombinant DNA sequence.]

[61. Seed from a transgenic plant according to Claim 60.]

[62. Progeny from a transgenic plant according to Claim 60.]

[63. Progeny from seed of a transgenic plant according to Claim 60.]

[64. Use of a transgenic plant according to Claim 43 for the preparation of a nutraceutical preparation for achieving a nutritional effect.]

[65. Use of a transgenic plant according to Claim 43 for the preparation of a pharmaceutical preparation for achieving a therapeutic effect.]

## GENETIC MANIPULATION OF ISOFLAVONOIDS

### TECHNICAL FIELD OF THE INVENTION

The invention relates to gene manipulation in plants.

### BACKGROUND OF THE INVENTION

5       The flavonoids are a major class of phenylpropanoid-derived plant natural products. Their fifteen carbon ( $C_6-C_3-C_6$ ) backbone can be arranged as a 1,3-diphenylpropane skeleton (flavonoid nucleus) or as a 1,2-diphenylpropane skeleton (isoflavonoid nucleus). Although 1,3-diphenylpropane flavonoid derivatives are almost ubiquitous among terrestrial plants, the 1,2-diphenylpropane isoflavonoids are  
10       restricted primarily to the Leguminosae, although they occur rarely in other families such as the Apocynaceae, Pinaceae, Compositae, and Moraceae (Tahara, S. and R. K. Ibrahim, 1995, "Prenylated isoflavonoids - an update," *Phytochemistry* 38: 1073-1094).

15       The limited taxonomic distribution of the isoflavonoids is directly related to the occurrence of the enzyme complex isoflavone synthase (IFS), which catalyzes the aryl migration reaction leading to the formation of an isoflavone from a flavanone. While flavanones are ubiquitous in higher plants, the IFS reaction, which is a two-step process specific for isoflavonoid biosynthesis (Kochs, G. and H. Grisebach, 1986, "Enzymic synthesis of isoflavones," *European J Biochem* 155: 311-318), is limited to  
20       the Leguminosae and the other diverse taxa in which isoflavonoids are occasionally found.

25       The presence of isoflavonoids provides several advantages to plants. One such advantage is provided by the function of isoflavonoids as antimicrobial phytoalexins in plant-microbe interactions. For example, the simple isoflavones daidzein and genistein act as initial precursors in the biosynthesis of various antimicrobial isoflavonoid phytoalexins in a wide variety of legumes (Dixon, R. A. and N. L. Paiva, 1995, "Stress-induced phenylpropanoid metabolism," *Plant Cell* 7: 1085-1097). Isoflavonoid compounds have been shown to accumulate in infected plant cells to

levels known to be antimicrobial *in vitro*. The temporal, spatial and quantitative aspects of accumulation are consistent with a role for these compounds in disease resistance (Rahe, J. E., 1973, "Occurrence and levels of the phytoalexin phaseollin in relation to delimitation at sites of infection of *Phaseolus vulgaris* by *Colletotrichum lindemuthianum*," *Canadian J Botany* 51: 2423-2430; Hadwiger, L. A. and D. M. Webster, 1984, "Phytoalexin production in five cultivars of pea differentially resistant to three races of *Pseudomonas syringae* pv. *pisi*," *Phytopathology* 74: 1312-1314; Long, et al., 1985, "Further studies on the relationship between glyceollin accumulation and the resistance of soybean leaves to *Pseudomonas syringae* pv. *glycinea*," *Phytopathology* 75: 235-239; Bhattacharyya, M. K. and E. W. B. Ward, 1987, "Biosynthesis and metabolism of glyceollin I in soybean hypocotyls following wounding or inoculation with *Phytophthora megasperma* f. sp. *glycinea*," *Physiol and Mol Plant Pathology* 31: 387-405). Moreover, it has been reported that many plant pathogens are much more sensitive to phytoalexins of non-host species than they are to the phytoalexins of their natural hosts, because they can often detoxify the host's phytoalexins. (VanEtten, et al., 1989, "Phytoalexin detoxification: importance for pathogenicity and practical implications," *An Rev Phytopathology* 27: 143-164).

Isoflavonoids also function in plant-microbe interactions in the establishment of bacterial or fungal symbioses with plants. Isoflavonoids have been reported to regulate bacterial nodulation genes. acting as a major *nod* gene inducer (Kosslak, et al., 1987, "Induction of *Bradyrhizobium japonicum* common *nod* genes by isoflavones isolated from *Glycine max*," *Proc Natl Acad Sci USA* 84: 7428-7432) and/or transcription activator (Dakora, et al., 1993, "Common bean root exudates contain elevated levels of daidzein and coumestrol in response to *Rhizobium* inoculation," *Mol Plant-Microbe Interact* 6: 665-668). Isoflavonoids have also been shown to have a role on the establishment of the symbiotic vesicular arbuscular mycorrhizal (VAM) association of the fungus *Glomus* with legume roots. (Kape, et al., 1992, "Legume root metabolites and VA-mycorrhiza development," *J Plant Physiol* 141: 54-60). Xie et al have reported that the isoflavonoids coumestrol, daidzein and genistein have small but significant stimulatory effects on the degree of mycorrhizal colonization of soybean, and that one effect of isoflavonoids on the



soybean mycorrhizal symbiosis could be via induction of nodulation factors from co-colonizing Rhizobia, since nod-factors have also been shown to stimulate fungal colonization (Xie, et al., 1995, "Rhizobial nodulation factors stimulate mycorrhizal colonization of nodulating and nonnodulating soybeans," *Plant Physiology* 108: 1519-1525).

In addition to the advantages that the presence of isoflavonoids confers to plants, a significant body of evidence indicates that dietary consumption of isoflavonoids can provide benefits to human health. Dietary isoflavones have been ascribed strong cancer chemopreventative activity in humans, and display a range of pharmacological activities suggestive of various other health promoting effects, including phytoestrogen activity as both estrogenic and anti-estrogenic agents (Coward, et al., 1993, "Genistein, daidzein, and their -glycoside conjugates: antitumor isoflavones in soybean foods from American and Asian diets," *J Agricultural and Food Chemistry* 41: 1961-1967; Martin, et al., 1996, "Interactions between phytoestrogens and human sex steroid binding protein," *Life Sciences* 58: 429-436); anticancer effects associated with phytoestrogenic activity (Lee, et al., 1991, "Dietary effects on breast-cancer risk in Singapore," *Lancet* 337: 1197-1200; Adlercreutz, et al., 1991, "Urinary excretion of lignans and isoflavonoid phytoestrogens in Japanese men and women consuming a traditional Japanese diet," *Am J Clin Nutr* 54: 1093-1100); anticancer effects associated with inhibition of several enzymes including DNA topoisomerase and tyrosine protein kinase (Akiyama, et al., 1987, "Genistein, a specific inhibitor of tyrosine-specific protein kinases," *J Biol Chem* 262: 5592-559; Uckun, et al., 1995, "Biotherapy of B-cell precursor leukemia by targeting genistein to CD19-associated tyrosine kinases," *Science* 267: 886-891); suppression of alcohol consumption (Keung, W. M. and B. L. Vallee, 1993, "Daidzin: A potent, selective inhibitor of human mitochondrial aldehyde dehydrogenase," *Proc Natl Acad Sci USA* 90: 1247-1251; Keung, et al., 1995, "Daidzin suppresses ethanol consumption by Syrian golden hamsters without blocking acetaldehyde metabolism," *Proc Natl Acad Sci USA* 92: 8990-8993); antioxidant activity (Arora, et al., 1998, "Antioxidant activities of isoflavones and their biological metabolites in a liposomal system," *Arch Biochem Biophys* 356: 133-141; Tikkanen, et

al., 1998, "Effect of soybean phytoestrogen intake on low density lipoprotein oxidation resistance," *Proc Natl Acad Sci USA* 95: 3106-3110); effects on calcium metabolism, some of which may be linked to protective effects against osteoporosis (Tomonaga, et al., 1992, "Isoflavonoids, genistein, PSI-tectorigenin, and orobol, increase cytoplasmic free calcium in isolated rat hepatocytes," *Biochem Biophys Res Com* 182: 894-899; Draper, et al., 1997, "Phytoestrogens reduce bone loss and bone resorption in oophorectomized rats," *J Nutr* 127: 1795-1799); and cardiovascular effects (Wagner, et al., 1997, "Dietary soy protein and estrogen replacement therapy improve cardiovascular risk factors and decrease aortic cholesteryl ester content in ovariectomized cynomolgus monkeys," *Metabolism - Clinical and Experimental* 46: 698-705).

At present, the only dietary sources of isoflavonoids for humans are certain legumes such as soybean or chickpea. The development of methods to genetically manipulate isoflavonoids in plants, either to widen the source of dietary isoflavonoids for humans, or to exploit the biological activities of isoflavonoids for plant protection and improvement, is wholly dependent on the availability of cloned genes encoding the various enzymes of isoflavonoid biosynthesis. Of these, the isoflavone synthase (IFS) complex constitutes the first committed reactions, and as such represents the means to introduce isoflavonoids into plants that do not possess the pathway.

In 1984, Hagmann and Grisebach provided the first evidence for the enzymatic conversion of flavanone to isoflavone (the IFS reaction) in a cell free system (Hagmann, M. and H. Grisebach, 1984, "Enzymatic rearrangement of flavanone to isoflavone," *FEBS Letters* 175: 199-202). They demonstrated that microsomes from elicitor-treated soybean cell suspension cultures could catalyze the conversion of 2(S)-naringenin to genistein, or of 2(S)-liquiritigenin to daidzein, in the presence of NADPH. The crude microsomal enzyme preparation, which was stable at -70°C but had a half-life of only 10 minutes at room temperature, was absolutely dependent on NADPH and molecular oxygen. It was subsequently shown that the reaction proceeded in two steps. The flavanone was converted in a cytochrome P450-catalyzed reaction requiring NADPH and O<sub>2</sub> to the corresponding 2-hydroxyisoflavanone. This

relatively unstable compound, which could, however, be identified by mass spectrometric analysis, then underwent dehydration to yield the isoflavone. The dehydration reaction appeared to be catalyzed by an enzyme present predominantly in the cytoplasmic supernatant, although it was not possible to remove all this activity from the microsomes. The corresponding 2-hydroxyisoflavanone spontaneously converted to genistein, for example, in methanol at room temperature. Kinetic analysis indicated that the 2-hydroxyisoflavanone was formed prior to genistein, consistent with its being an intermediate in isoflavone formation. (Kochs, G. and H. Grisebach, 1986, "Enzymic synthesis of isoflavones," *European J Biochem* 155: 311-318).

Involvement of cytochrome P450 in the 2-hydroxyisoflavanone synthase reaction was confirmed by inhibition by CO, replacing O<sub>2</sub> with N<sub>2</sub>, and examining the effects of a range of known P450 inhibitors of which ancymidol was the most effective. The enzyme co-migrated with the endoplasmic reticulum markers cinnamate 4-hydroxylase (another cytochrome P450) and cytochrome b5 reductase on Percoll gradients. The enzyme is stereoselective, and (2*R*)-naringenin is not a substrate. (Kochs, G. and H. Grisebach, 1986, "Enzymic synthesis of isoflavones," *European J Biochem* 155: 311-318).

The origin of the 2-hydroxyl group was determined from studies on the IFS present in microsomes from elicited cell cultures of *Pueraria lobata*. <sup>18</sup>O from <sup>18</sup>O<sub>2</sub> was incorporated into the 2-hydroxyl group, resulting in a 2-hydroxyisoflavanone with molecular ion shifted by two mass units, whereas there was no corresponding shift in the molecular ion of daidzein, consistent with the subsequent dehydration reaction (Hashim, et al., 1990, "Reaction mechanism of oxidative rearrangement of flavanone in isoflavone biosynthesis," *FEBS Letters* 271: 219-222). The currently accepted model for the reaction pathway of IFS as illustrated in Fig. 1, therefore, involves P450-catalyzed hydroxylation coupled to aryl migration, a reaction with mechanistic similarities to the well described proton migration mechanism of some P450 reactions (Hakamatsuka, et al., 1991, "P-450-dependent oxidative rearrangement in isoflavone

biosynthesis: reconstitution of P-450 and NADPH:P450 reductase," *Tetrahedron* 47: 5969-5978).

Currently, there have been no reports on purification to homogeneity or molecular cloning of the cytochrome P450 of the IFS complex because of the extreme  
5 lability of the enzyme. The 2-hydroxyisoflavanone synthase cytochrome P450 from *Pueraria* has been solubilized with Triton X-100, and partially purified by DEAE-Sephadex chromatography; the enzymatic reaction could be reconstituted by addition of NADPH cytochrome P450 reductase that separated from the hydroxylase on the ion exchange column (Hakamatsuka, et al., 1991, *Tetrahedron* 47: 5969-5978). A 2-  
10 hydroxyisoflavanone dehydratase has been purified from elicitor-treated *P. lobata* cells, and has been shown to be a soluble monomeric enzyme of subunit Mr 38,000 (Hakamatsuka, et al., 1998, "Purification of 2-hydroxyisoflavanone dehydratase from the cell cultures of *Pueraria lobata*," *Phytochemistry* 49: 497-505). It is not yet clear whether this enzyme physically associates with the P450 hydroxylase catalyzing the  
15 aryl migration, or even whether this activity is essential for isoflavone formation *in planta* in view of the spontaneous conversion of 2-hydroxyisoflavanone to isoflavone.

Flavanone is a potential substrate for more than one type of hydroxylation reaction at the 2-position. Thus, elicitor-treated cell cultures of alfalfa and *Glycyrrhiza echinata* have been shown to accumulate the dibenzoylmethane licodione  
20 (Kirikae, et al., 1993, "Biosynthesis of a dibenzoylmethane, licodione, in cultured alfalfa cells induced by yeast extract," *Biosci Biotech Biochem* 57: 1353-1354). Licodione synthase is, by classical criteria, a cytochrome P450, the activity of which is induced by yeast elicitor in *Glycyrrhiza* cells (Otani, et al., 1994, "Licodione synthase, a cytochrome P450 monooxygenase catalyzing 2-hydroxylation of 5-deoxyflavanone,  
25 in cultured *Glycyrrhiza echinata* L. cells," *Plant Physiol* 105: 1427-1432). The reaction it catalyzes involves 2-hydroxylation of flavanone followed by hemiacetal opening instead of aryl migration, and the reaction was thought to have mechanistic similarities to the flavone synthase II enzyme previously characterized from soybean (Kochs, G. and H. Grisebach, 1987, "Induction and characterization of a NADPH-  
30 dependent flavone synthase from cell cultures of soybean," *Z. Naturforsch* 42C: 343-

348). A gene encoding the flavone synthase II/licodione synthase from *Glycyrrhiza* has been cloned (Akashi, et al., 1998, "Identification of a cytochrome P450 cDNA encoding (2S)-flavanone 2-hydroxylase of licorice (*Glycyrrhiza echinata* L.: *Fabaceae*) which represents licodione synthase and flavone synthase II," *FEBS Letters* 5 431: 287-290), and a different cytochrome P450 gene encoding flavone synthase II has recently been cloned from *Gerbera hybrida* (Martens, S. and G. Forkmann, "Cloning and expression of flavone synthase II from *Gerbera* hybrids," *Plant J* 20: 611-618).

Although the reactions catalyzed by IFS are critical for the formation of all isoflavonoids in plants, there have been no previous reports of the isolation of genes encoding components of isoflavone synthase, although genes encoding most of the other enzymes of the isoflavonoid pathway, including downstream enzymes converting simple isoflavones to antimicrobial phytoalexins, have been characterized (Dixon, et al., 1995, "The isoflavonoid phytoalexin pathway: from enzymes to genes to transcription factors," *Physiologia Plantarum* 93: 385-392). Thus, the 10 unavailability of isoflavone synthase genes has made it heretofore impossible to utilize the downstream genes for regulating isoflavonoid concentrations in legumes and other plants that do have the isoflavonoid pathway, or for engineering antimicrobial and pharmacologically active isoflavonoids in transgenic plants of species that do not have the isoflavonoid pathway. 15

20 Genes encoding the enzyme catalyzing the first step of the isoflavone synthase reaction have now been isolated and purified from soybean and *Medicago truncatula* (barrel medic).

## BRIEF DESCRIPTION OF THE DRAWINGS

25 Fig. 1 depicts the currently accepted model for the reaction pathway of IFS wherein the flavanone is converted in a cytochrome P450-catalyzed reaction requiring NADPH and O<sub>2</sub> to the corresponding 2-hydroxyisoflavanone which then undergoes dehydration to yield the isoflavone.

Fig. 2 depicts the nucleotide sequence of soybean CYP93C1v2.

Fig. 3 depicts the amino acid sequence of soybean CYP93C1v2 compared to licorice CYP93B1.

Fig. 4 depicts the nucleotide sequence of *Medicago truncatula* mtIFSE3.

Fig. 5 depicts the amino acid sequence of *Medicago truncatula* mtIFSE3 compared to soybean CYP93C1v2.

Fig. 6A and Fig. 6B depict HPLC traces of extracts from pooled tissues (leaves, shoots, flowers) of *Arabidopsis thaliana* ecotype Columbia harboring an empty tDNA vector (Fig. 6A) and *Arabidopsis thaliana* ecotype Columbia harboring the soybean CYP93C1v2 cDNA sequence (Fig. 6B). The empty vector transformed line contains a number of flavonol glycosides and other phenolic compounds that are also present in the CYP93C1v2 transformed line. These compounds were identified as (a) rhamnose (Rha)- glucose (Glc)- quercetin (Q), (b) uncharacterized conjugate of Q, (c) Rha-Glc-Rha-Kaempferol (K), (d) Glu-Rha-Q, (e) Rha-Rha-Q, (f) Glc-Rha-K, (g) sinapic acid, (h) Rha-Rha-K. Three additional compounds were observed in the CYP93C1v2 transformed line (Fig. 6B), and labeled "1," "2" and "3." Fig. 6C depicts a total ion chromatogram of partially purified peaks 2 and 3, and the insets show the specific ions generated from these compounds. Peak 2 has a parental molecular mass ion of 579.5 consistent with genistein conjugated to a glucose-rhamnose disaccharide, and two further mass ions of 417.5 and 271.3, representing Rha-genistein and free genistein, respectively. Peak 3, which has a parental molecular ion of mass 417.5, is thereby identified as Rha-genistein.

Fig. 7A and Fig. 7B depict HPLC traces of the same extracts as shown in Fig. 6A (empty-vector transformed) and Fig. 6B, (CYP93C1v2 transformed), but following digestion with  $\beta$ -glucosidase. Peaks 2 and 3 remained at the same retention time as in Fig. 6A and 6B. However, Peak 1 disappeared, and was replaced with a new Peak 4 of much later retention time. Fig. 7C shows the total ion chromatograph of purified Peak 4, and the inset shows the parental molecular ion, with mass of 271.2, consistent with Peak 4 being free genistein. Fig. 7D shows a total ion chromatograph, and the parental molecular ion, of an authentic sample of genistein.

Fig. 8A , 8B, 8C and 8D are high performance liquid chromatography (HPLC) chromatograms depicting the presence of new peaks at RT 29.96 and 37.7 min representing the presence of the isoflavone daidzein formed from the flavanone liquiritigenin, or the isoflavone genistein formed from the flavanone naringenin, in insect cell microsomes expressing CYP93C1v2. Fig. 8A depicts the presence of NADPH during incubation with liquiritigenin. Fig. 8B depicts the absence of NADPH during incubation with liquiritigenin. Fig. 8C depicts the presence of NADPH during incubation with naringenin. Fig. 8D depicts the lack of a reaction when soybean CYP93E expressed in insect cells is incubated with liquiritigenin in the presence of NADPH.

Fig. 9A and Fig. 9B are mass spectra of BSTFA (*N,O*-bis(trimethylsilyl) trifluoroacetamide) derivatives. Fig. 9A depicts the mass spectrum of the BSTFA derivative of the product of the reaction catalyzed by CYP93C1v2 in insect cells using liquiritigenin as substrate, and Fig. 9B shows the mass spectrum of the BSTFA derivative of an authentic sample of daidzein .

## SUMMARY OF THE INVENTION

In one aspect, the invention is a method for introducing into a naturally non-isoflavonoid-producing plant species the enzyme catalyzing the aryl migration of a flavanone to form an isoflavanone intermediate or an isoflavone, comprising introducing a DNA segment encoding the enzyme into the plant to form a transgenic plant, wherein the transgenic plant expresses the DNA segment under the control of a suitable constitutive or inducible promoter when the transgenic plant is exposed to conditions which permit expression. The DNA segment can comprise isolated genomic DNA or recombinant DNA. Preferably, the DNA segment is a CYP93C gene. An exemplary DNA segment from a soybean CYP93C gene consists essentially of the sequence from about nucleotide 36 to about nucleotide 1598 of the sequence depicted in SEQ ID NO:1. Another preferred DNA segment comprises a *Medicago truncatula* homolog of a CYP93C gene, more preferably, the sequence from about nucleotide 92 to about nucleotide 1657 of the sequence depicted in SEQ ID NO:4. Plants transformed by this method may also preferably express chalcone synthase,

chalcone reductase, and chalcone isomerase genes to cause in vivo formation of daidzein or a daidzein derivative, and the chalcone synthase, chalcone reductase, and chalcone isomerase genes may also be transgenes. Plants transformed by this method may also preferably further comprise downstream genes, for example, isoflavone *O*-methyltransferase, isoflavone 2'-hydroxylase, isoflavone reductase, and vestitone reductase, to metabolize a formed isoflavone to biologically active isoflavonoid derivatives or conjugates. The plant can comprise isoflavone 4'-*O*-methyl-transferase to cause formation of biochanin A or a biochanin A derivative from the isoflavanone intermediate. An exemplary flavanone substrate for this transformation method is liquiritigenin and/or naringenin.

In another aspect, the present invention is a method for increasing the level of isoflavonoid compounds in naturally isoflavonoid-producing plants comprising introducing a DNA segment encoding the enzyme catalyzing the aryl migration of a flavanone to yield an isoflavonoid to form a transgenic plant, wherein the transgenic plant expresses the DNA segment under the control of a suitable constitutive or inducible promoter when the transgenic plant is exposed to conditions which permit expression. With this method, the resulting isoflavonoid can be an isoflavanone intermediate, an isoflavone, an isoflavone derivative, and an isoflavone conjugate. The DNA segment can comprise isolated genomic DNA or recombinant DNA. Preferably, the DNA segment is a CYP93C gene. An exemplary DNA segment from a soybean CYP93C gene consists essentially of the sequence from about nucleotide 36 to about nucleotide 1598 of the sequence depicted in SEQ ID NO:1. Another preferred DNA segment comprises a *Medicago truncatula* homolog of a CYP93C gene, more preferably, the sequence from about nucleotide 92 to about nucleotide 1657 of the sequence depicted in SEQ ID NO:4. An exemplary flavanone substrate for this transformation method is liquiritigenin and/or naringenin.

In another aspect, the invention is a method for synthesizing an isoflavanone intermediate or an isoflavone from a flavanone by expressing a recombinant CYP93C gene segment in a suitable bacterial, fungal, algal, or insect cell system. An exemplary gene segment consists essentially of the sequence from about nucleotide 36



to about nucleotide 1598 of the sequence depicted in SEQ ID NO:1. Another exemplary gene segment consists essentially of the sequence from about nucleotide 92 to about nucleotide 1657 of the sequence depicted in SEQ ID NO:4.

In another aspect, the invention is a method of reducing the levels of isoflavonoid compounds in a naturally isoflavonoid-producing plant comprising introducing and expressing an antisense or gene silencing construct that contains an intact CYP93C gene or segments thereof into the plant. An exemplary gene consists essentially of the sequence from about nucleotide 36 to about nucleotide 1598 of the sequence depicted in SEQ ID NO:1. Another exemplary gene consists essentially of the sequence from about nucleotide 92 to about nucleotide 1657 of the sequence depicted in SEQ ID NO:4.

In another aspect, the invention is a naturally non-isoflavonoid-producing plant cell transformed by introducing a DNA segment encoding the enzyme catalyzing the aryl migration of a flavanone to form an isoflavanone intermediate or an isoflavone, wherein the transformed plant cell expresses the DNA segment under the control of a suitable constitutive or inducible promoter when exposed to conditions which permit expression. The DNA segment can comprise isolated genomic DNA or recombinant DNA. Preferably, the DNA segment is a CYP93C gene. An exemplary DNA segment from a soybean CYP93C gene consists essentially of the sequence from about nucleotide 36 to about nucleotide 1598 of the sequence depicted in SEQ ID NO:1. Another preferred DNA segment comprises a *Medicago truncatula* homolog of a CYP93C gene, more preferably, the sequence from about nucleotide 92 to about nucleotide 1657 of the sequence depicted in SEQ ID NO:4. Plants transformed by this method may also preferably express chalcone synthase, chalcone reductase, and chalcone isomerase genes to cause in vivo formation of daidzein or a daidzein derivative, and the chalcone synthase, chalcone reductase, and chalcone isomerase genes may also be transgenes. Plants transformed by this method may also preferably further comprise downstream genes, for example, isoflavone *O*-methyltransferase, isoflavone 2'-hydroxylase, isoflavone reductase, and vestitone reductase, to metabolize a formed isoflavanone intermediate to biologically active isoflavonoid

derivatives or conjugates. The plant can comprise isoflavone 4'-O-methyl-transferase to cause formation of biochanin A or a biochanin A derivative from the isoflavanone intermediate.

In another aspect, the invention is a naturally isoflavonoid-producing plant cell transformed by introducing a DNA segment encoding the enzyme catalyzing the aryl migration of a flavanone to yield an isoflavonoid to form a transformed plant cell, wherein the transformed plant cell expresses the DNA segment under the control of a suitable constitutive or inducible promoter when exposed to conditions which permit expression. With this method, the resulting isoflavonoid can be an isoflavanone intermediate, an isoflavone, an isoflavone derivative, and an isoflavone conjugate. The DNA segment can comprise isolated genomic DNA or recombinant DNA. Preferably, the DNA segment is a CYP93C gene. An exemplary DNA segment from a soybean CYP93C gene consists essentially of the sequence from about nucleotide 36 to about nucleotide 1598 of the sequence depicted in SEQ ID NO:1. Another preferred DNA segment comprises a *Medicago truncatula* homolog of a CYP93C gene, more preferably, the sequence from about nucleotide 92 to about nucleotide 1657 of the sequence depicted in SEQ ID NO:4.

In another aspect, the invention is a transgenic plant cell having reduced levels of isoflavonoid compounds, the plant cell transformed by introducing an antisense or gene silencing construct that contains an intact CYP93C gene or segments thereof into the plant cell. An exemplary gene consists essentially of the sequence from about nucleotide 36 to about nucleotide 1598 of the sequence depicted in SEQ ID NO:1. Another exemplary gene consists essentially of the sequence from about nucleotide 92 to about nucleotide 1657 of the sequence depicted in SEQ ID NO:4.

In another aspect, the invention is an isolated gene or DNA segment comprising a portion which encodes a cytochrome P450 that can catalyze the aryl migration of a flavanone to yield an isoflavanone intermediate or an isoflavone, wherein the portion consists essentially of about nucleotide 36 to about nucleotide 1598 of the sequence depicted in SEQ ID NO:1. An exemplary gene is the soybean gene encoding the enzyme catalyzing the aryl migration of liquiritigenin. Another

exemplary gene is the soybean gene encoding the enzyme catalyzing the aryl migration of naringenin.

5 In another aspect, the invention is a protein encoded by a portion of an isolated gene or DNA segment which encodes a cytochrome P450 that can catalyze the aryl migration of a flavanone to yield an isoflavanone intermediate or an isoflavone, wherein the portion consists essentially of about nucleotide 36 to about nucleotide 1598 of the sequence depicted in SEQ ID NO:1.

10 In another aspect, the invention is an isolated gene or DNA segment comprising a portion which encodes a cytochrome P450 that can catalyze the aryl migration of a flavanone to yield an isoflavanone intermediate or an isoflavone, wherein the portion is a *Medicago truncatula* homolog of a CYP93C gene. An exemplary gene or DNA segment consists essentially of about nucleotide 92 to about nucleotide 1657 of the sequence depicted in SEQ ID NO:4. An exemplary gene is the *Medicago truncatula* gene encoding the enzyme catalyzing the aryl migration of  
15 liquiritigenin. Another exemplary gene is the *Medicago truncatula* gene encoding the enzyme catalyzing the aryl migration of naringenin.

In another aspect, the invention is a protein encoded by a portion of an isolated gene or a DNA segment which encodes a cytochrome P450 that can catalyze the aryl migration of a flavanone to yield an isoflavanone intermediate or an isoflavone,  
20 wherein the portion is a *Medicago truncatula* homolog of a CYP93C gene.

In yet another aspect, the invention is a food comprising edible transgenic plant material capable of being ingested for its nutritional value, wherein the transgenic plant has been transformed with an isolated gene or DNA segment which encodes a cytochrome P450 that can catalyze the aryl migration of a flavanone to yield an  
25 isoflavanone intermediate or an isoflavone, and wherein the transgenic plant exhibits increased levels of an isoflavonoid when compared to the level of the isoflavonoid in plants of the same species which do not comprise the isolated gene or DNA segment.

In yet another aspect, the invention is a food comprising at least one isoflavonoid, wherein the isoflavonoid is isolated from a transgenic plant transformed

with an isolated gene or DNA segment which encodes a cytochrome P450 that can catalyze the aryl migration of a flavanone to yield an isoflavanone intermediate or an isoflavone, and wherein the transgenic plant exhibits increased levels of an isoflavonoid when compared to the level of the isoflavonoid in plants of the same species which do not comprise the isolated gene or DNA segment.

In yet another aspect, the invention is a composition comprising at least a portion of a transgenic plant transformed with an isolated gene or DNA segment which encodes a cytochrome P450 that can catalyze the aryl migration of a flavanone to yield an isoflavanone intermediate or an isoflavone, wherein the transgenic plant exhibits increased levels of an isoflavonoid when compared to the level of the isoflavonoid in plants of the same species which do not comprise the isolated gene or DNA segment, and wherein the composition is suitable for ingestion as a food stuff, a nutritional supplement, an animal feed supplement, or a nutraceutical.

In yet another aspect, the invention is a composition comprising an isoflavonoid suitable for administration as a food stuff, a nutritional supplement, an animal feed supplement, a nutraceutical, or a pharmaceutical, wherein the isoflavonoid is isolated from at least a portion of a transgenic plant transformed with an isolated gene or DNA segment which encodes a cytochrome P450 that can catalyze the aryl migration of a flavanone to yield an isoflavanone intermediate or an isoflavone, and wherein the transgenic plant exhibits increased levels of an isoflavonoid when compared to the level of the isoflavonoid in plants of the same species which do not comprise the isolated gene or DNA segment.

In yet another aspect, the invention is a method of increasing the nutritional value of a plant by transforming the plant with an isolated gene or DNA segment which encodes a cytochrome P450 that can catalyze the aryl migration of a flavanone to yield an isoflavanone intermediate or an isoflavone, wherein the transgenic plant exhibits increased levels of an isoflavonoid when compared to the level of the isoflavonoid in plants of the same species which do not comprise the isolated gene or DNA segment.

In yet another aspect, the invention is a method of using a transgenic plant transformed with an isolated gene or DNA segment which encodes a cytochrome P450 that can catalyze the aryl migration of a flavanone to yield an isoflavanone intermediate or an isoflavone, wherein the transgenic plant exhibits an increased level of an isoflavonoid when compared to the level of the isoflavonoid in plants of the same species which do not comprise the isolated gene or DNA segment, to provide a nutraceutical benefit to a human or animal administered the isoflavonoid. The isoflavonoid can be administered by ingestion of at least a portion of the plant. The isoflavonoid can also be administered by ingestion of a composition comprising an isoflavonoid isolated from the plant.

In yet another aspect, the invention is a method of using an isoflavonoid isolated from a transgenic plant transformed with an isolated gene or DNA segment which encodes a cytochrome P450 that can catalyze the aryl migration of a flavanone to yield an isoflavanone intermediate or an isoflavone, wherein the transgenic plant exhibits an increased level of an isoflavonoid when compared to the level of the isoflavonoid in plants of the same species which do not comprise the isolated gene or DNA segment, to provide a pharmaceutical benefit to a patient administered the isoflavonoid.

In yet another aspect, the invention is a method of increasing disease resistance in a plant by transforming the plant with an isolated gene or DNA segment which encodes a cytochrome P450 that can catalyze the aryl migration of a flavanone to yield an isoflavanone intermediate or an isoflavone, wherein the transgenic plant exhibits an increased level of an isoflavonoid when compared to the level of the isoflavonoid in plants of the same species which do not comprise the isolated gene or DNA segment.

In yet another aspect, the invention is a method of increasing nodulation efficiency of a leguminous plant by transforming the plant with an isolated gene or DNA segment which encodes a cytochrome P450 that can catalyze the aryl migration of a flavanone to yield an isoflavanone intermediate or an isoflavone, wherein the transgenic plant exhibits an increased level of an isoflavonoid released from the roots

when compared to the level of the isoflavonoid released from the roots of plants of the same species which do not comprise the isolated gene or DNA segment.

In yet another aspect, the invention is a transgenic leguminous plant exhibiting increased nodulation efficiency transformed with an isolated gene or DNA segment which encodes a cytochrome P450 that can catalyze the aryl migration of a flavanone to yield an isoflavanone intermediate or an isoflavone, wherein the transgenic plant exhibits an increased level of an isoflavonoid released from the roots when compared to the level of the isoflavonoid released from the roots of plants of the same species which do not comprise the isolated gene or DNA segment.

In yet another aspect, the invention is a method of increasing bacterial or fungal symbiosis in a plant by transforming the plant with an isolated gene or DNA segment which encodes a cytochrome P450 that can catalyze the aryl migration of a flavanone to yield an isoflavanone intermediate or an isoflavone, wherein the transgenic plant exhibits an increased level of an isoflavonoid when compared to the level of the isoflavonoid in plants of the same species which do not comprise the isolated gene or DNA segment.

In yet another aspect, the invention is a transgenic plant exhibiting increased bacterial or fungal symbiosis transformed with an isolated gene or DNA segment which encodes a cytochrome P450 that can catalyze the aryl migration of a flavanone to yield an isoflavanone intermediate or an isoflavone, wherein the transgenic plant exhibits an increased level of an isoflavonoid when compared to the level of the isoflavonoid in plants of the same species which do not comprise the isolated gene or DNA segment.

In yet another aspect, the invention is a transgenic plant comprising at least one recombinant DNA sequence encoding a cytochrome P450 that can catalyze the aryl migration of a flavanone to yield an isoflavanone intermediate or an isoflavone, wherein the transgenic plant exhibits an increased level of an isoflavonoid when compared to the level of the isoflavonoid in plants of the same species which do not comprise the recombinant DNA sequence.

In yet another aspect, the invention is seed from a transgenic plant comprising at least one recombinant DNA sequence encoding a cytochrome P450 that can catalyze the aryl migration of a flavanone to yield an isoflavanone intermediate or an isoflavone, wherein the transgenic plant exhibits an increased level of an isoflavonoid when compared to the level of the isoflavonoid in plants of the same species which do not comprise the recombinant DNA sequence.

In yet another aspect, the invention is progeny from a transgenic plant comprising at least one recombinant DNA sequence encoding a cytochrome P450 that can catalyze the aryl migration of a flavanone to yield an isoflavanone intermediate or an isoflavone, wherein the transgenic plant exhibits an increased level of an isoflavonoid when compared to the level of the isoflavonoid in plants of the same species which do not comprise the recombinant DNA sequence.

In yet another aspect, the invention is progeny from seed of a transgenic plant comprising at least one recombinant DNA sequence encoding a cytochrome P450 that can catalyze the aryl migration of a flavanone to yield an isoflavanone intermediate or an isoflavone, wherein the transgenic plant exhibits an increased level of an isoflavonoid when compared to the level of the isoflavonoid in plants of the same species which do not comprise the recombinant DNA sequence.

In yet another aspect, the invention is use of a transgenic plant transformed with an isolated gene or DNA segment which encodes a cytochrome P450 that can catalyze the aryl migration of a flavanone to yield an isoflavanone intermediate or an isoflavone, wherein the transgenic plant exhibits an increased level of an isoflavonoid when compared to the level of the isoflavonoid in plants of the same species which do not comprise the isolated gene or DNA segment, for the preparation of a nutraceutical preparation for achieving a nutritional effect.

In yet another aspect, the invention is use of a transgenic plant transformed with an isolated gene or DNA segment which encodes a cytochrome P450 that can catalyze the aryl migration of a flavanone to yield an isoflavanone intermediate or an isoflavone, wherein the transgenic plant exhibits an increased level of an isoflavonoid

when compared to the level of the isoflavonoid in plants of the same species which do not comprise the isolated gene or DNA segment, for the preparation of a pharmaceutical preparation for achieving a therapeutic effect.

## DETAILED DESCRIPTION

5 One aspect of the present invention is an isolated gene which encodes the first step of the isoflavone synthase reaction: a cytochrome P450 that can catalyze the aryl migration of a flavanone to yield an isoflavone. Genes and corresponding cDNA of the soybean or *Medicago truncatula* CYP93 family have been isolated. The enzymes encoded by the genes of the present invention are isoflavone synthases (IFS) and can  
10 catalyze the aryl migration of a flavanone to yield an isoflavone either directly or through the intermediacy of a 2-hydroxyisoflavanone. One isolated soybean gene is classified as *CYP93C1v2*.

Cytochrome P450 enzymes belong to a large superfamily of enzymes that are abundant in every living organism. The P450 nomenclature committee has  
15 determined that each P450 should carry a "CYP" designation and arbitrarily divided the superfamily into families (alphabetical designation), subfamilies (numerical designation) and allelic variants ("v" plus numerical designation) based on amino acid identity of >40%, >55%, and >97%, respectively (Nelson, et al. 1993. "The P450  
20 superfamily update on new sequences, gene mapping, accession numbers, early trivial names of enzymes, and nomenclature," *DNA Cell Biol* 12:1). Thus, *CYP93C1v2* is a variant of the first described P450 belonging to the third subfamily (C) of the ninety-third P450 family.

Utilizing the procedures presented herein, any plant known to produce isoflavonoids may also serve as sources of suitable DNA, or coding sequences may be  
25 synthesized in vitro based on the sequences for the IFS genes of the present invention. CYP93 family members can also be obtained from other plant species by polymerase chain reaction amplification methods known to those skilled in the art, using primer sequences corresponding to regions of nucleotide conservation between CYP93 family members. Furthermore, the genes of the present invention are defined by their



catalytic activity: the aryl migration of a flavanone to yield an isoflavone. The gene sequences presented as SEQ ID NO:1 and SEQ ID NO:4 are exemplary, and it is understood that modifications to these genes which do not alter the catalytic activity of its encoded protein fall within the scope of the present invention. While a preferred IFS gene contains the entire open reading frame, portions of or the entire 5' and 3' untranslated regions as well as portions of the vector sequence can also be present. With the isolation and functional identification of these isoflavone synthase (IFS) genes that encode the first key step in isoflavone formation, the aryl migration reaction, it is now possible to introduce the isoflavonoid pathway into all plant species, including those that do not naturally possess this pathway.

Another aspect of the present invention is a genetically modified plant which has been transformed with a gene of the present invention. For example, when the *CYP93C1v2* gene is transferred into the model plant *Arabidopsis thaliana*, which does not naturally produce isoflavonoids, the isoflavone genistein accumulates as a series of glycoconjugates (Example 1). This demonstrates that the genes of the present invention can be genetically engineered into plants which do not naturally contain the isoflavonoid pathway, and the transgenic plants can then produce isoflavonoids, resulting in plants with improved disease resistance and/or value added health benefits for humans. In the present invention, unless otherwise stated, as used herein, the term "plant" or "progeny" includes plant parts, plant tissue, plant cells, plant protoplasts, plant cell tissue cultures from which plants can be regenerated, plant calli, plant clumps, explants, plant cells that are intact in plants, or parts of plants, such as embryos, pollen, ovules, flowers, capsules, stems, leaves, seeds, roots, root tips, and the like. Furthermore, the present invention includes the IFS genes expressed in various parts of the plant, e.g., in aerial portions of the plant useful for increasing disease resistance or production of health promoting isoflavonoid nutraceuticals, in seeds useful for increasing levels of isoflavones and their conjugates, or in roots useful for increasing disease resistance or production of nodulation gene inducing isoflavones.

In another aspect, the present invention is a method of improving disease resistance and a transgenic plant with increased disease resistance. By transforming a plant which does not naturally make isoflavones with an IFS gene of the present invention, disease resistance can be genetically engineered into the plant by providing  
5 the necessary enzyme to convert its natural flavanones into isoflavonoids. The introduction and subsequent expression of an IFS gene of the present invention into a crop species which naturally possesses the isoflavonoid pathway results in increased levels of the isoflavonoid defense compounds.

In another aspect, the present invention is a method of increasing levels of  
10 isoflavonoids that might be beneficial to the establishment of bacterial or fungal symbioses with plants and a transgenic plant with an increased capacity for symbiotic association with bacteria or fungi. Bacterial nodulation can be stimulated in transgenic leguminous plants by expression of an IFS gene of the present invention and decreased by expression of antisense constructs or constructs designed to promote  
15 gene silencing that contain an intact IFS gene or segments thereof. Mycorrhizal colonization of leguminous plants can also be increased through the introduction and expression of an IFS gene of the present invention.

In yet another aspect, the present invention is a method of producing isoflavonoid compounds in plants or any other organism to be used in nutraceuticals or  
20 pharmaceuticals to confer human or animal health benefits. Edible transgenic plants high in isoflavonoids can be utilized as food for humans and animals. Edible compositions high in isoflavonoids can also be made by incorporation of the transgenic plants or plant materials, or by incorporation of isoflavonoids isolated from the transgenic plants. Compositions useful for administration as a food stuff, a  
25 nutritional supplement, an animal feed supplement, a nutraceutical, or a pharmaceutical can be made by incorporation of the transgenic plants or plant materials, or by incorporation of isoflavonoids isolated from the transgenic plants. The nutritional value of a plant can be increased by transforming the plant with an IFS gene of the present invention and, as a result, accumulating high amounts of  
30 isoflavonoids in the plant.

The soybean IFS gene of the present invention was isolated and purified according to the detailed procedures outlined in Example 2. The DNA sequence is shown in SEQ ID NO:1 and Fig. 2, and the encoded protein sequence of the isolated soybean CYP93C clone is shown in SEQ ID NO:2 and Fig. 3. For comparison, Fig. 3  
5 also shows the protein sequence alignment between the isolated CYP93C clone (SEQ ID NO:2) and CYP93B1 (SEQ ID NO:3), the licorice licodione synthase.

The DNA and protein sequences of the soybean CYP93C1 open reading frame were deposited in the Genbank data base under accession # AF022462. The deposition was made by Siminszky, Dewey and Corbin, and the sequence described as  
10 representing a gene induced in soybean in response to herbicide safeners. However, the function of the gene was not known and there was no understanding that it could be involved in isoflavonoid biosynthesis at the time the deposit was made (Siminszky, B., Corbin, F.T., Ward, E.R., Fleischmann, T.J. and Dewey, R.E., 1999, "Expression of a soybean cytochrome P450 monooxygenase cDNA in yeast and tobacco enhances  
15 the metabolism of phenylurea herbicides." *Proc. Natl. Acad. Sci. USA* 96: 1750-1755). The sequence of the clone characterized herein differs from CYP93C1 in three nucleotide substitutions in the open reading frame that change proline 140 to leucine, threonine 156 to isoleucine, and glutamate 295 to lysine. Thus, the soybean gene identified herein has been classified as CYP93C1v2.

20 The cDNA insert from CYP93C1v2 was used to probe 240,000 phage plaques from a *Medicago truncatula* root cDNA library (van Buuren, M.L., I.E. Maldonado-Mendoza, A.T. Trieu, L.A. Blaylock, and M.J. Harrison, 1999, "Novel genes induced during an arbuscular mycorrhizal (AM) symbiosis formed between *Medicago truncatula* and *Glomus versiforme*," *Mol. Plant-Microbe Interact.* 12, 171-181). Five  
25 positive plaques were purified, in vivo excised, and sequenced. A full length clone designated mtIFSE3 was completely sequenced on both strands, and shown to encode the *Medicago truncatula* homolog of soybean CYP93C1. The nucleotide sequence of mtIFSE3 is shown in SEQ ID NO:4 and Fig. 4, and the protein sequence, in SEQ ID NO:5. An alignment between the protein sequences of mtIFSE3 and CYP93C1v2 is  
30 shown in Fig. 5.

An IFS gene of the soybean or *Medicago truncatula* CYP93C subfamily or corresponding cDNA sequence, the open reading frame of which encodes a cytochrome P450 that can catalyze the aryl migration of a flavanone to yield an isoflavone, either directly or through the intermediacy of a 2-hydroxyisoflavanone, can be used to introduce the isoflavonoid pathway into any plant species that does not naturally possess this pathway. Soybean CYP93C1v2 acts on the flavanones liquiritigenin to yield daidzein, and naringenin to yield genistein. Liquiritigenin is only formed in plants that possess the enzyme chalcone reductase (CHR) (Welle, R. and Grisebach, H., 1989, "Phytoalexin synthesis in soybean cells: elicitor induction of reductase involved in biosynthesis of 6'-deoxychalcone." *Arch Biochem Biophys* 272: 97-102), and a form of chalcone isomerase that is active against 2',4,4'-trihydroxychalcone, the product of the co-action of chalcone synthase (CHS) with CHR (Dixon, R.A., Blyden, E.R., Robbins, M.P., van Tunen, A.J. and Mol, J.N.M., 1988, "Comparative biochemistry of chalcone isomerases." *Phytochemistry* 27: 2801-2808). Such genes are common in legumes, but not in most other plant families. Thus, to form daidzein in transgenic plants that do not possess the isoflavonoid pathway, it would be necessary to introduce three new genes, namely CHR, to co-act with CHS to form 2',4,4'-trihydroxychalcone, a suitable CHI to convert 2',4,4'-trihydroxychalcone to liquiritigenin, and IFS, assuming that the 2-hydroxyisoflavanone intermediate can spontaneously dehydrate in planta, a phenomenon that is demonstrated below. Without CHR present, no liquiritigenin would be formed, and IFS would only be able to act on naringenin to yield, assuming spontaneous dehydration of the 2-hydroxyisoflavanone, genistein.

The IFS genes of the present invention can be introduced into non-leguminous plants such as by standard *Agrobacterium*-based or biolistic transformation procedures (Horsch, et al., 1985, "A simple and general method for transferring genes into plants," *Science* 227:1229-1231; and Klein, et al., 1988, "Stable genetic transformation of intact *Nicotiana* cells by the particle bombardment process," *Proc Natl Acad Sci USA* 85:8502-8505). Both procedures require the construction of a plasmid vector containing a desirable transcriptional promoter driving expression of the gene of interest (in this case IFS), followed by a transcriptional terminator and a

selectable marker gene for resistance, such as to an antibiotic or a herbicide. The biolistic procedure coats metal particles with plasmid DNA containing the gene of interest and places them on a micro carrier disk. Using the biolistic apparatus, the particles are physically propelled into plant tissue. The plant tissue is then put under selection (e.g., antibiotic or herbicide) followed by regeneration. The two *Agrobacterium*-based procedures are "in planta" and "ex-planta", respectively. Both procedures require the above gene construct to be placed into a T-DNA vector, which is then transferred into *Agrobacterium tumefaciens*. The in planta procedure places the transformed *Agrobacterium* in the presence of plant material (flower or meristem) and the plants are allowed to seed followed by selection (e.g., antibiotic or herbicide) during germination. The ex-planta procedure also places *Agrobacterium* in the presence of plant material (callus, cell culture, leaf disk, hypocotyl) which is placed directly under selection (e.g., antibiotic or herbicide) followed by regeneration.

Thus, the isoflavonoid pathway can be introduced into any plant species that does not possess the enzyme catalyzing the IFS reaction by expressing the IFS gene in transgenic plants under the control of a suitable constitutive or inducible promoter.

**Example 1: Transformation of *Arabidopsis thaliana* with Soybean CYP93C1V2**

Soybean CYP93C1v2 cDNA was placed in the binary plant transformation vector pCHF3, in which it is under control of the cauliflower mosaic virus 35S promoter, using standard recombinant DNA methods (Sambrook, et al. 1989. *Molecular Cloning. A Laboratory Manual*, 2nd Ed, Cold Spring Harbor Laboratory Press, New York). The gene was then transformed into plants of the crucifer, *Arabidopsis thaliana* ecotype Columbia, using *Agrobacterium tumefaciens* and a standard floral infiltration procedure (Clough, S.J. and Bent, A.F., 1998, "Floral dip: a simplified method for *Agrobacterium*-mediated transformation of *Arabidopsis thaliana*". *Plant J* 16: 735-743). Transgenic plants were selected by germinating the seedlings on kanamycin, and those surviving selection were allowed to set seed. T<sub>2</sub> seedlings expressing CYP93C1v2 were identified by standard DNA and RNA gel blot analysis (Sambrook, et al. 1989. *Molecular cloning. A Laboratory Manual*, 2nd Ed,

Cold Spring Harbor Laboratory Press, New York), and analyzed for accumulation of genistein in leaves by HPLC analysis, according to a method developed to profile the flavonoid components of *Arabidopsis* leaves (Graham, T.L., 1998, "Flavonoid and flavonol glycoside metabolism in *Arabidopsis*". *Plant Physiol Biochem* 36: 135-144).

5           Figure 6A shows a typical HPLC trace of a leaf extract from an untransformed plant. The major components are glycosides (containing glucose and rhamnose) of the flavonols kaempferol and quercetin. Plants harboring the soybean CYP93C1v2 gene showed an additional three peaks on HPLC analysis (Fig. 6B), indicated by the arrows labeled as "1," "2" and "3." No free genistein, free 2-hydroxyisoflavanone or 2-  
10   hydroxyisoflavanone conjugates were observed. However, following treatment of extracts with almond  $\beta$ -glucosidase (Fig 7B), one of the new peaks disappeared, and free genistein was now observed, consistent with the peak being a glucoside of genistein. LC-MS analysis confirmed the identities of the new compounds as a glucoside of genistein, glucose-rhamnose-genistein, and rhamnose-genistein (Figs. 6C  
15   and 7C and 7D, insets). Therefore, expression of CYP93C1v2 in transgenic *Arabidopsis* leads to formation of genistein with no requirement for an enzyme to catalyze the dehydration of the presumed 2-hydroxyisoflavanone intermediate. *Arabidopsis* plants then modify the genistein by exactly the same chemistry they use to conjugate their endogenous flavonols, namely by conjugation to glucose and  
20   rhamnose. Transgenic production of conjugates of genistein are suitable for nutraceutical applications, because genistein is also glycosylated in soybean, its natural dietary source (Graham, T.L., 1991, "Flavonoid and isoflavonoid distribution in developing soybean seedling tissues and in seed and root exudates." *Plant Physiol* 95: 594-603).

25           In addition to introducing the isoflavonoid pathway into plants that do not possess this pathway, the level of isoflavonoid compounds can be controlled in plants that do possess the pathway by manipulating the level of expression of the IFS gene. Increasing the levels of isoflavonoid compounds in leguminous plants by expression of the IFS gene of the present invention in transgenic plants under the control of a  
30   suitable constitutive or inducible promoter can be accomplished by standard methods

such as *Agrobacterium*-based or biolistic transformation methods known in the art. Alternatively, the level of isoflavonoid compounds in plants can be reduced by expression of antisense constructs or constructs designed to promote gene silencing that contain an intact IFS gene, or segments thereof, in transgenic plants using

5 methods known in the art. (Bourque, J.E., 1995, "Antisense strategies for genetic manipulations in plants," *Plant Science* 105:125-149; and Angell, S. M. and D. C. Baulcombe, 1997, "Consistent gene silencing in transgenic plants expressing a replicating potato virus X RNA," *EMBO J* 16:3675-3684). Antisense constructs for gene silencing are constructed by placing the whole or part of the cDNA in a three

10 prime to five prime orientation behind a desirable transcriptional promoter and ahead of a transcriptional terminator in a plasmid vector. The vector may be used for biolistic transformation or the new antisense gene may be transferred to a T-DNA vector for *Agrobacterium*-based transformation. The actual mechanism of silencing by antisense constructs is unknown. Homology-dependent gene silencing or co-

15 suppression requires the over-expression of a homologous gene; therefore, to achieve co-suppression a construct is made using a strong promoter, the gene of interest (in this case IFS) and a transcriptional terminator. The gene should be transferred to plants as described above. Gene silencing is an epigenetic phenomenon that may or may not occur with a particular gene construct. When it does occur, the inhibition of

20 gene expression can be greater than with the antisense approach.

Isoflavones can be synthesized from flavanones, utilizing recombinant IFS expressed in any suitable bacterial, fungal, algal, or insect cell system. For example, naringenin is extracted in large amounts from grapefruits. A CYP93C1 enzyme can be used convert naringenin to 2,5,7,4'-tetrahydroxyisoflavanone, which spontaneously

25 converts to the valuable nutraceutical genistein under weak acid conditions. Furthermore, daidzin can be synthesized from liquiritigenin utilizing recombinant CYP93C1 and an isoflavone glucosyltransferase (Köster, J. and W. Barz, 1981, "UDP-Glucose: isoflavone 7-O-glucosyltransferase from roots of chick pea (*Cicer arietinum* L.)." *Arch Biochem Biophys* 212: 98-104).

**Example 2: Methodology Used to Isolate and Identify IFS cDNA Clones**

In an attempt to obtain cDNA clones encoding IFS, a functional genomics approach was followed. IFS activity is present in soybean seeds, which accumulate daidzein and genistein. Furthermore, IFS activity can be induced in soybean tissues in response to infection with *Phytophthora infestans*, associated with the accumulation of the isoflavonoid phytoalexin glyceollin (Bhattacharyya, M. K. and E. W. B. Ward, 1987, "Biosynthesis and metabolism of glyceollin I in soybean hypocotyls following wounding or inoculation with *Phytophthora megasperma* f. sp. *glycinea*," *Physiol Mol Plant Path* 31: 387-405). It was also known that an enzyme catalyzing a similar reaction to IFS, namely the 2-hydroxylation of flavanone but without aryl migration, belongs to the CYP93B1 subclass of cytochrome P450s (Akashi, et al, 1998, "Identification of a cytochrome P450 cDNA encoding (2S)-flavanone 2-hydroxylase of licorice (*Glycyrrhiza echinata* L.: *Fabaceae*) which represents licodione synthase and flavone synthase II," *FEBS Letters* 431: 287-290). We therefore searched an expressed sequence tag (EST) database of partial soybean sequences obtained by mass sequencing of two cDNA libraries: a *Phytophthora*-infected hypocotyl cDNA library (48 hours after infection) and a mid to late developmental stage seed library. Nine candidate P450 sequences were identified, of which three belonged to the CYP93 family. DNA probes were made from the EST clones of the three CYP93 candidates and were used to probe an RNA blot of transcripts from alfalfa suspension cells at various times after exposure to yeast elicitor, a treatment known to induce IFS activity at the onset of isoflavonoid phytoalexin accumulation (Kessmann, et al., 1990, "Stress responses in alfalfa (*Medicago sativa* L.) III. Induction of medicarpin and cytochrome P450 enzyme activities in elicitor-treated cell suspension cultures and protoplasts," *Plant Cell Reports* 9: 38-41). One P450 probe cross-hybridized and detected alfalfa transcripts that were strongly induced by elicitation. This probe was derived from a clone with high homology to soybean CYP93C1 as described below, and the insert in the EST clone was full length. The insert was excised and then cloned into the baculovirus expression system for functional identification by heterologous expression in insect cells (Pauli, H. H. and T. M. Kutchan, 1998, "Molecular cloning and functional heterologous expression of two alleles encoding (S)-N-methylcoclaurine 3'-



hydroxylase (CYP80B1), a new methyl jasmonate-inducible cytochrome P-450-dependent mono-oxygenase of benzylisoquinoline alkaloid biosynthesis," *The Plant J* 13: 793-801).

The carbon monoxide difference spectrum of microsomes isolated from insect cells expressing the soybean CYP93C clone indicated the presence of expressed cytochrome P450, as seen from an absorption peak at 450 nm that was not present in similar spectra from insect microsomes originating from cells transformed with a control vector. Unlabeled liquiritigenin was then fed to the microsomes in the presence of NADPH. The substrate remained unconverted in microsomes from cells harboring the control vector. However, in microsomes expressing the CYP93C clone, a new peak of RT 29.96 min was observed by high performance liquid chromatography (Fig 8A). The amount of this peak was reduced 10-fold if NADPH was omitted from the incubations (Fig. 8B). The UV spectrum of the product, obtained by diode array detection, was identical to that of authentic daidzein ( $\lambda_{\max}$  248 nm, sh 302 nm,  $\lambda_{\min}$  222 nm). The product was collected, derivatized, and analyzed by GC-MS. The mass spectrum of the BSTFA derivative was identical to that of an authentic sample of daidzein (Fig. 9). Microsomes containing the CYP93C clone also metabolized naringenin to yield genistein, although somewhat less efficiently than the reaction with liquiritigenin (Fig. 8C). Insect cell microsomes expressing a different soybean cytochrome P450 cDNA, CYP93E, did not convert liquiritigenin to daidzein when incubated in the presence of NADPH (Fig. 8D). These results indicate that the soybean CYP93C encodes IFS.

### Example 3: Method of Increasing Dietary Isoflavonoid Intake

Transgenic tomato plants are produced by the introduction of *CYP93C1v2* via standard *Agrobacterium*-based procedures. In a preferred embodiment, the *CYP93C1v2* coding sequence is under control of a gene promoter giving specific expression in the fruit. Progeny containing the coding region of the *CYP93C1v2* gene are selected at the seedling stage by standard polymerase chain reaction and/or DNA blot analysis known to those skilled in the art. Plants scoring positive for possession of the transgene are grown to fruiting, and fruit analyzed for the presence of

isoflavones by the HPLC methods shown in Fig. 7 and Fig. 8 of the present invention. Fruit harvested from the transgenic tomato plants are ingested to increase the dietary intake of isoflavonoids.

5 It is to be understood that the above description is of preferred exemplary embodiments of the invention and is intended to be illustrative of the invention, but is not to be construed to limit the scope of the invention in any way. Modifications may be made in the structural features of the invention without departing from the scope of the invention.

10 In summary, isoflavones can now be genetically engineered to provide potential human health benefits of dietary isoflavones and to increase disease resistance in plants. Isoflavones can now be produced in transgenic plants species in which isoflavones do not naturally occur, i.e., in species other than legumes. For example, engineering constitutive production of daidzein and/or genistein or their conjugates into tomato, potato, corn, or other popular components of the human diet,  
15 leads to human health benefits, such as reduced cancer risk, reduced incidence of osteoporosis, and treatment for alcoholism. Alternatively, introducing infection-inducible isoflavonoid biosynthesis into non-legumes qualitatively complements these plants' phytoalexin defenses against microbial pathogens, whereas over-expression of the isoflavonoid pathway in legumes quantitatively increases this defense response.  
20 Finally, modifying the extent of production of isoflavonoids in legume roots positively impacts nodulation efficiency and therefore plant yield.

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**WE CLAIM:**

1. A method for introducing into a naturally non-isoflavonoid-producing plant species the enzyme catalyzing the aryl migration of a flavanone to form an isoflavanone intermediate or an isoflavone, comprising:

- 5 introducing a DNA segment encoding said enzyme into said plant to form a transgenic plant, wherein said transgenic plant expresses said DNA segment under the control of a suitable constitutive or inducible promoter when said transgenic plant is exposed to conditions which permit expression.

2. The method of Claim 1, wherein chalcone synthase, chalcone reductase, and chalcone isomerase genes are also expressed in said plant to cause in vivo formation of daidzein or a daidzein derivative.

3. The method of Claim 2, wherein said plant is further transformed to comprise said chalcone synthase, chalcone reductase, and chalcone isomerase genes.

4. The method of Claim 1 or 2, wherein said plant further comprises downstream genes to metabolize said formed isoflavanone intermediate or isoflavone to biologically active isoflavonoid derivatives or conjugates.

5. The method of Claim 4, wherein said downstream gene is selected from the group consisting of isoflavone *O*-methyltransferase, isoflavone 2'-hydroxylase, isoflavone reductase, and vestitone reductase.

6. The method of Claim 5, wherein said plant comprises downstream gene 4'-*O*-methyltransferase to form biochanin A or a biochanin A derivative.

7. A method for increasing the level of isoflavonoid compounds in naturally isoflavonoid-producing plants comprising:  
introducing a DNA segment encoding the enzyme catalyzing the aryl migration of a flavanone to yield an isoflavonoid to form a transgenic plant, wherein said transgenic plant expresses said  
5 DNA segment under the control of a suitable constitutive or inducible promoter when said transgenic plant is exposed to conditions which permit expression.

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8. The method of Claim 7, wherein said isoflavonoid is selected from the group consisting of an isoflavonone intermediate, an isoflavone, an isoflavone derivative, and an isoflavone conjugate.

9. The method of Claim 1, 7 or 8, wherein said DNA segment comprises isolated genomic DNA.

10. The method of Claim 1, 7 or 8, wherein said DNA segment comprises recombinant cDNA.

11. The method of Claim 7-10, wherein said DNA segment comprises CYP93C gene.

12. The method of Claim 7-10, wherein said DNA segment is a *Medicago truncatula* homolog of a CYP93C gene.

13. The method of Claim 1-12, wherein said flavanone is liquiritigenin.

14. The method of Claim 1-12, wherein said flavanone is naringenin.

15. A method for synthesizing an isoflavanone intermediate or an isoflavone from a flavanone by expressing a recombinant CYP93C gene segment in a suitable bacterial, fungal, algal, or insect cell system.

16. A method of reducing the levels of isoflavonoid compounds in a naturally isoflavonoid-producing plant comprising introducing and expressing an antisense or gene silencing construct that contains an intact CYP93C gene or segments thereof into said plant.

17. The method of Claim 1, 11, 15 or 16, wherein said gene consists of the sequence from nucleotide 36 to nucleotide 1598 of the sequence depicted in SEQ ID NO:1.

18. The method of Claim 1, 12, 15 or 16, wherein said gene consists of the sequence from nucleotide 92 to nucleotide 1657 of the sequence depicted in SEQ ID NO:4.

19. A naturally non-isoflavonoid producing plant cell transformed by introducing a DNA segment encoding the enzyme catalyzing the aryl migration of a flavanone to form an isoflavanone intermediate or an isoflavone, wherein said transgenic plant cell expresses said DNA segment under the control of a suitable constitutive or inducible promoter when exposed to conditions which permit expression.

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20. The plant cell of Claim 19, wherein chalcone synthase, chalcone reductase, and chalcone isomerase genes are also expressed in said plant to cause in vivo formation of daidzein or a daidzein derivative.

21. The plant cell of Claim 20, wherein said plant cell is further transformed to comprise said chalcone synthase, chalcone reductase, and chalcone isomerase genes.

22. The plant cell of Claim 19-20, wherein said plant cell further comprises downstream genes to metabolize said formed isoflavanone intermediate or isoflavone to biologically active isoflavonoid derivatives or conjugates.

23. The plant cell of Claim 22, wherein said downstream gene is selected from the group consisting of isoflavone *O*-methyltransferase, isoflavone 2'-hydroxylase, isoflavone reductase, and vestitone reductase.

24. The plant cell of Claim 23, wherein said plant cell comprises downstream gene 4'-*O*-methyltransferase to form biochanin A or a biochanin A derivative.

25. A naturally isoflavonoid-producing plant cell transformed by introducing a DNA segment encoding the enzyme catalyzing the aryl migration of a flavanone to yield an isoflavonoid to form a transformed plant cell, wherein said transformed plant cell expresses said DNA segment under the control of a suitable constitutive or inducible promoter when exposed to conditions which permit expression.

26. The plant cell of Claim 25, wherein said isoflavonoid is selected from the group consisting of an isoflavanone intermediate, an isoflavone, an isoflavone derivative, and an isoflavone conjugate.

27. The plant cell of Claim 19, 25 or 26, wherein said DNA segment comprises isolated genomic DNA.

28. The plant cell of Claim 19, 25 or 26, wherein said DNA segment comprises recombinant cDNA.

29. The plant cell of Claim 19 or 25-28, wherein said DNA segment comprises CYP93C gene.

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Patent Specialist

30. The plant cell of Claim 19 or 25-28, wherein said DNA segment is a *Medicago truncatula* homolog of a CYP93C gene.

31. A transgenic plant cell having reduced levels of isoflavonoid compounds, said plant cell transformed by introducing an antisense or gene silencing construct that contains an intact CYP93C gene or segments thereof into said plant cell.

32. The plant cell of Claim 29 or 31, wherein said gene consists of the sequence from nucleotide 36 to nucleotide 1598 of the sequence depicted in SEQ ID NO:1.

33. The plant cell of Claim 30 or 31, wherein said gene consists of the sequence from nucleotide 92 to nucleotide 1657 of the sequence depicted in SEQ ID NO:4.

34. An isolated gene or DNA segment comprising a portion which encodes a cytochrome P450 of the CYP93 family that can catalyze the aryl migration of a flavanone to yield an isoflavanone intermediate or an isoflavone, wherein said portion consists of nucleotide 36 to nucleotide 1598 of the sequence depicted in SEQ ID NO:1.

35. The gene or DNA segment of Claim 34, wherein said gene is the soybean gene encoding the enzyme catalyzing the aryl migration of liquiritigenin.

36. The gene or DNA segment of Claim 34, wherein said gene is the soybean gene encoding the enzyme catalyzing the aryl migration of naringenin.

37. A protein encoded by a portion of an isolated gene or DNA segment which encodes a cytochrome P450 that can catalyze the aryl migration of a flavanone to yield an isoflavanone intermediate or an isoflavone, wherein said portion consists of nucleotide 36 to nucleotide 1598 of the sequence depicted in SEQ ID NO:1.

38. An isolated gene or DNA segment comprising a portion which encodes a cytochrome P450 that can catalyze the aryl migration of a flavanone to yield an isoflavanone intermediate or an isoflavone, wherein said portion is a *Medicago truncatula* homolog of a CYP93C gene.

39. The gene or DNA segment of Claim 38 consisting of nucleotide 92 to nucleotide 1657 of the sequence depicted in SEQ ID NO:4.

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Karl Schmid

40. The gene or DNA segment of Claims 38 or 39, wherein said gene is the *Medicago truncatula* gene encoding the enzyme catalyzing the aryl migration of liquiritigenin.

41. The gene or DNA segment of Claims 38 or 39, wherein said gene is the *Medicago truncatula* gene encoding the enzyme catalyzing the aryl migration of naringenin.

42. A protein encoded by a portion of an isolated gene or DNA segment which encodes a cytochrome P450 that can catalyze the aryl migration of a flavanone to yield an isoflavanone intermediate or an isoflavone, wherein said portion is a *Medicago truncatula* homolog of a CYP93C gene.

43. A transgenic plant cell transformed with an isolated gene or DNA segment which encodes a cytochrome P450 that can catalyze the aryl migration of a flavanone to yield an isoflavanone intermediate or an isoflavone, wherein said transgenic plant cell exhibits increased levels of an isoflavonoid when compared to the level of said isoflavonoid in plant cells of the same species which do not comprise said isolated gene or DNA segment.

44. A food comprising edible transgenic plant material capable of being ingested for its nutritional value, wherein said transgenic plant comprises plant cells according to claim 43.

45. A method of preparing a food comprising at least one isoflavonoid comprising: transforming a plant according to the method of claim 1 or 7, wherein said transgenic plant exhibits increased levels of an isoflavonoid when compared to the level of said isoflavonoid in plants of the same species which do not comprise said DNA segment, isolating said isoflavonoid and incorporating into said food.

46. A composition comprising at least a portion of a transgenic plant according to claim 43, wherein said composition is suitable for ingestion as a food stuff, a nutritional supplement, an animal feed supplement, or a nutraceutical.

47. A method of preparing a composition comprising an isoflavonoid suitable for administration as a food stuff, a nutritional supplement, an animal feed supplement, a nutraceutical, or a pharmaceutical, comprising: transforming a plant according to the method of claim 1 or 7, wherein said transgenic plant exhibits increased levels of an isoflavonoid when compared to the level of said isoflavonoid in

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plants of the same species which do not comprise said DNA segment, isolating said isoflavonoid and incorporating into said compositions.

48. A method of using a transgenic plant according to claim 43 to provide a nutraceutical benefit to a human or animal administered said isoflavonoid.

49. The method of Claim 48, wherein said isoflavonoid is administered by ingestion of at least a portion of said plant.

50. The method of Claim 48, wherein said isoflavonoid is administered by ingestion of a composition comprising an isoflavonoid isolated from said plant.

51. A method for making a pharmaceutical preparation, comprising:  
transforming a plant according to the method of claim 1 or 7, wherein said transgenic plant exhibits increased levels of an isoflavonoid when compared to the level of said isoflavonoid in plants of the same species which do not comprise said DNA segment, isolating said isoflavonoid and formulating said isoflavonoid to form a pharmaceutical preparation.

52. A method of transforming a plant with an isolated gene or DNA segment which encodes a cytochrome P450 that can catalyze the aryl migration of a flavanone to yield an isoflavanone intermediate or an isoflavone, wherein said transgenic plant exhibits increased levels of an isoflavonoid when compared to the level of said isoflavonoid in plants of the same species which do not comprise said isolated gene or DNA segment.

53. A method of Claim 52, wherein the nutritional value of said plant is increased.

54. A method of Claim 52, wherein the disease resistance in said plant is increased.

55. A method of Claim 52, wherein bacterial or fungal symbiosis in said plant is increased.

56. A method of claim 52, wherein said plant is a leguminous plant.

57. A method of claim 56, wherein the nodulation efficiency of said plant is increased.

58. A leguminous transgenic plant exhibiting increased nodulation efficiency, wherein said transgenic plant is transformed according to the method of Claim 52.



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Patented September 11, 2001

59. A transgenic plant of Claim 43 exhibiting an increased level of bacterial or fungal symbiosis.

60. A transgenic plant comprising at least one recombinant DNA sequence encoding a cytochrome P450 that can catalyze the aryl migration of a flavanone to yield an isoflavanone intermediate or an isoflavone, wherein said transgenic plant exhibits an increased level of an isoflavonoid when compared to the level of said isoflavonoid in plants of the same species

5 which do not comprise said recombinant DNA sequence.

61. Seed from a transgenic plant according to Claim 60.

62. Progeny from a transgenic plant according to Claim 60.

63. Progeny from seed of a transgenic plant according to Claim 60.

64. Use of a transgenic plant according to Claim 43 for the preparation of a nutraceutical preparation for achieving a nutritional effect.

65. Use of a transgenic plant according to Claim 43 for the preparation of a pharmaceutical preparation for achieving a therapeutic effect.

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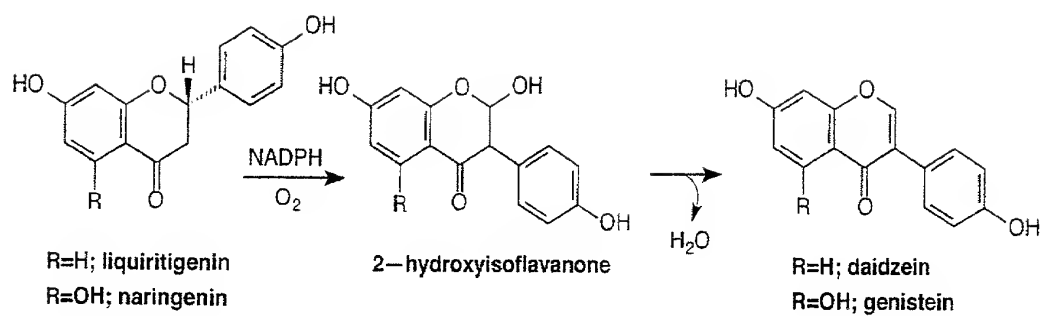


Fig. 1

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151 AGCCTCGTCT TCCCTTCATA GGACACCTTC ATCTCTTAAA AGACAAACTT  
201 CTCCACTACG CACTCATCGA CCTCTCCAAA AAACATGGTC CCTTATTCTC  
251 TCTCTACTTT GGCTCCATGC CAACCGTTGT TGCTCCACA CCAGAATTGT  
301 TCAAGCTCTT CCTCCAAACG CACGAGGCAA CTTCTTCAA CACAAGGTTT  
351 CAAACCTCAG CCATAAGACG CCTCACCTAT GATAGCTCAG TGGCCATGGT  
401 TCCCTTCGGA CCTTACTGGA AGTTCGTGAG GAAGCTCATC ATGAACGACC  
451 TTCTCAACGC CACCACTGTA AACAAGTTGA GGCTTTGAG GACCAACAG  
501 ATCCGCAAGT TCCTTAGGGT TATGGCCAA GGCGCAGAG CACAGAAGCC  
551 CCTTGACTTG ACCGAGGAGC TTCTGAAATG GACCAACAGC ACCATCTCCA  
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651 CTTAAGATCT TTGGCGAATA CAGCCTCACT GACTTCATCT GGCCATTGAA  
701 GCATCTCAAG GTTGGAAGT ATGAGAAGAG GATCGACGAC ATCTTGAACA  
751 AGTTCGACCC TGTCGTTGAA AGGGTCATCA AGAAGCGCCG TGAGATCGTG  
801 AGGAGGAGAA AGAACGGAGA GGTGTTGAG GGTGAGGTCA GCGGGTTTTT  
851 CCTTGACACT TTGCTTGAAT TCGCTGAGGA TGAGACCATG GAGATCAAAA  
901 TCACCAAGGA CCACATCAAG GGTCTTGTTG TCGACTTTTT CTGGCAGGA  
951 ACAGACTCCA CAGCGGTGGC AACAGAGTGG GCATTGGCAG AACTCATCAA  
1001 CAATCCTAAG GTGTGGAAA AGGCTCGTGA GGAGGTCTAC AGTGTTGTGG  
1051 GAAAGGACAG ACTTGTGGAC GAAGTTGACA CTCAAACCT TCCTTACATT  
1101 AGAGCAATCG TGAAGGAGAC ATTCCGCATG CACCCGCCAC TCCAGTGGT  
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1201 AGGGAGCATT GATTCTCTTC AATGTATGGC AAGTAGGAAG AGACCCCAA  
1251 TACTGGGACA GACCATCGGA GTTCCGTCCT GAGAGGTTCC TAGAGACAGG  
1301 GGCTGAAGGG GAAGCAGGGC CTCTTGATCT TAGGGGACAA CATTTTCAAC  
1351 TTCTCCCAT TGGGTCTGGG AGGAGAATGT GCCCTGGAGT CAATCTGGCT  
1401 ACTTCGGGAA TGGCAACACT TCTTGATCT CTTATTGAGT GCTTCGACTT  
1451 GCAAGTGCTG GGTCCACAAG GACAGATATT GAAGGTGGT GACGCCAAAG  
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1551 GTCTGTGTTT CACTTGCAAG GATCGGCGTT GCATCTAAAC TCCTTTCTTA  
1601 ATTAAGATCA TCGTCATCAT CATCATATGT AATATTTACT TTTTGTGTGT  
1651 TGATAATCAT CATTTCAATA AGGTCTCATT CATCTACTTT TTATGAAGTA  
1701 TATAAGCCCT TCCATGC

Fig. 2

**Fig. 3**

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```
1   CAACACCTAA GAGTAACTAA TAAGAACTTT CTTTCTACTT CTTAGTATAC
51  TTAACAACCTT AAGTAAATAT ACTACAAAGA AGCTATACAC CATGTTGGTG
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151 AACACCTACT GCAAAATCAA AGGCTCTTCG CCACCTTCCA AATCCACCAA
201 GCCCTAAACC ACGTCTTCCA TTCATAGGTC ATCTTCACCT TTTGGATAAC
251 CCACTTCTTC ACCACACTCT TATCAAGTTA GGAAAGCGTT ATGGCCCTTT
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1751 GAAGTGTTGT AACTTGTAAT TGCATACAAA ATCTATAAAT TTTATCATCC
1801 TATTCATTAT T
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Fig. 4

[illegible]

**Fig. 5**

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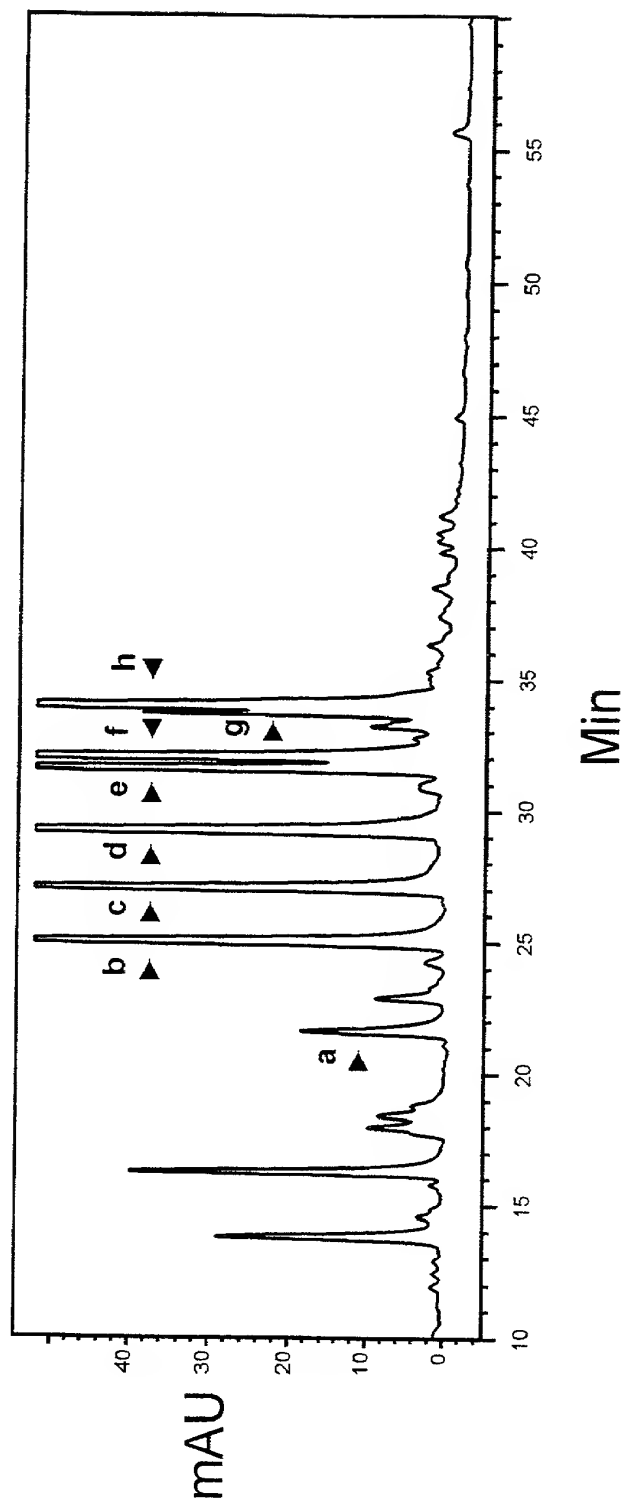


Fig. 6A

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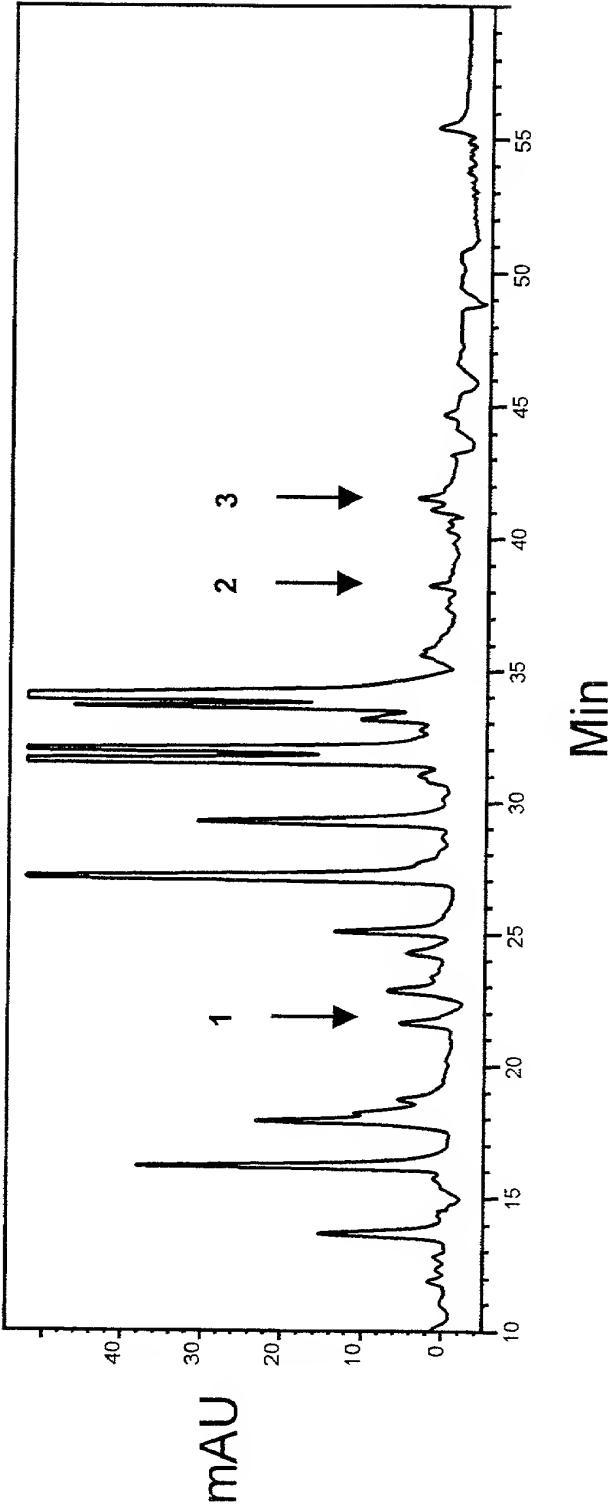


Fig. 6B



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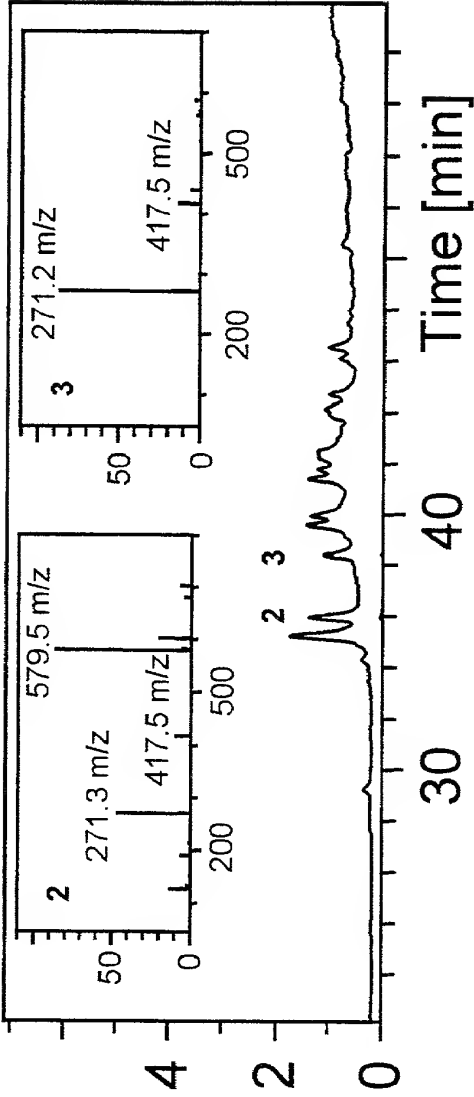


Fig. 6C

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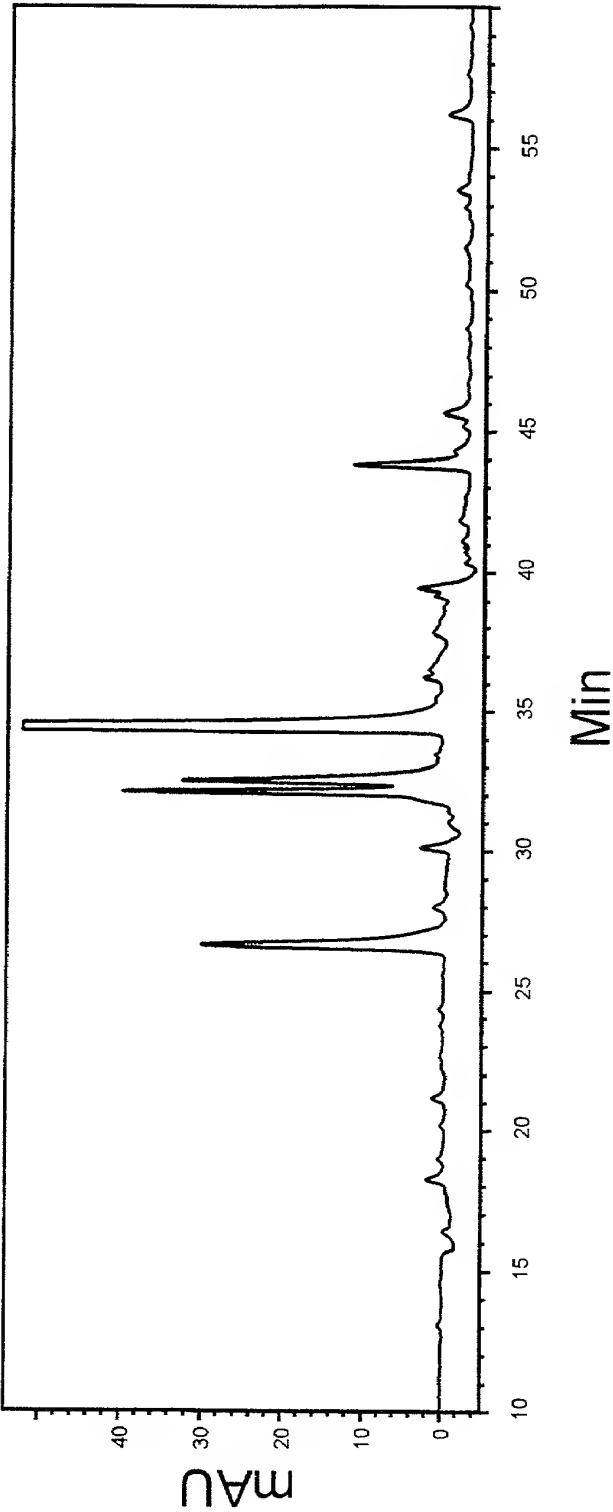


Fig. 7A

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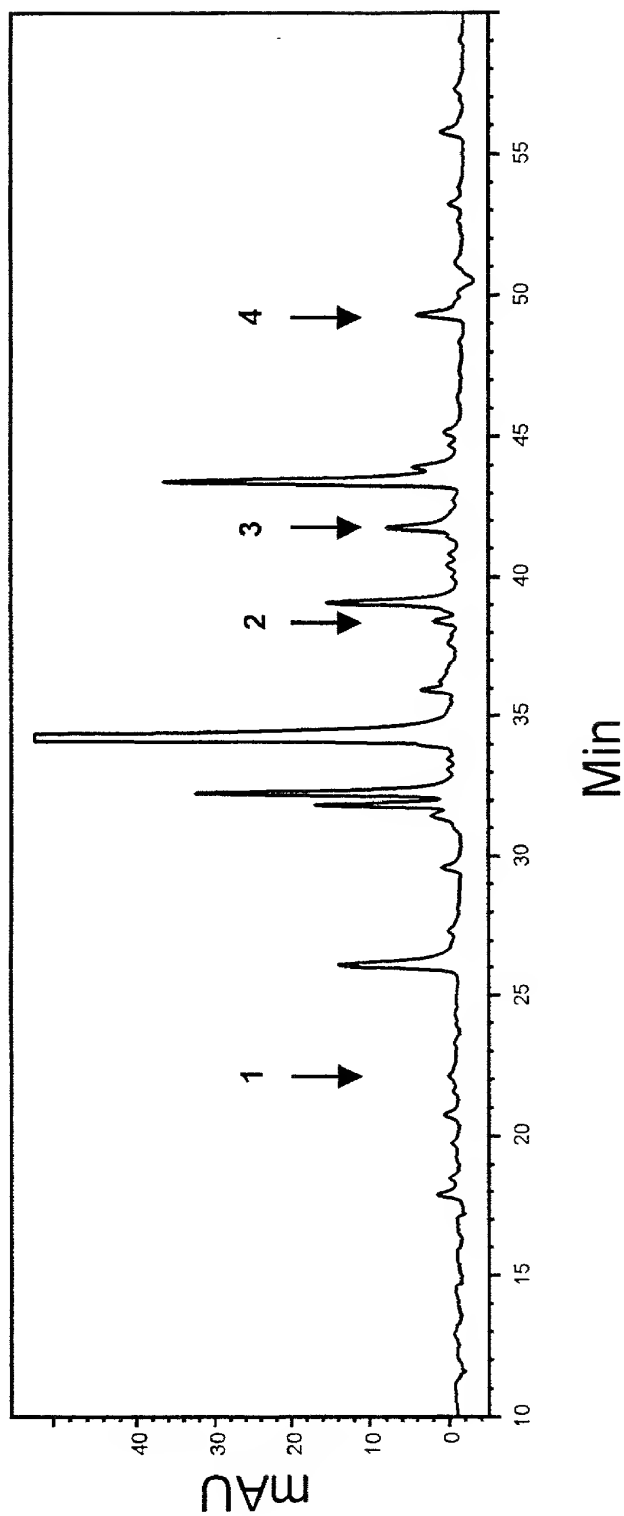


Fig. 7B

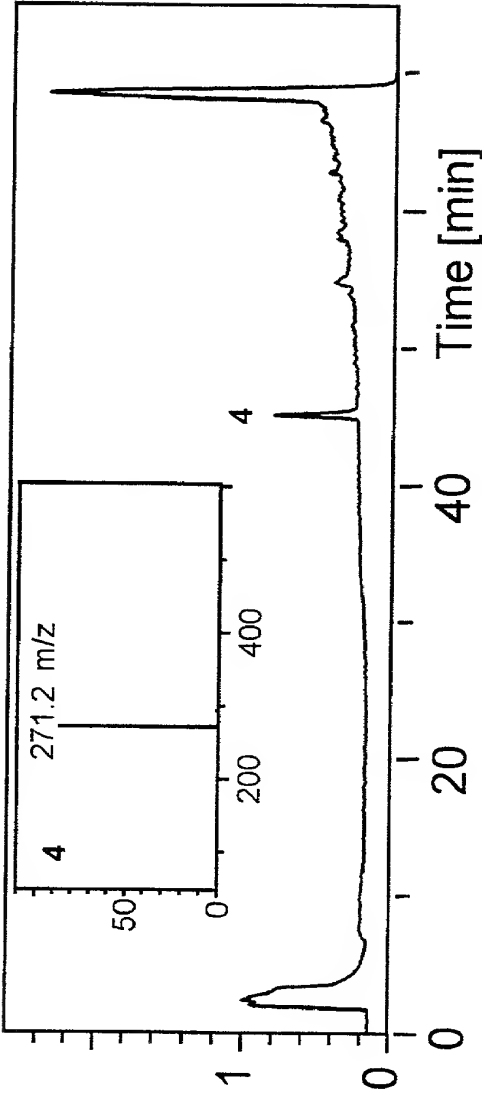


Fig. 7C

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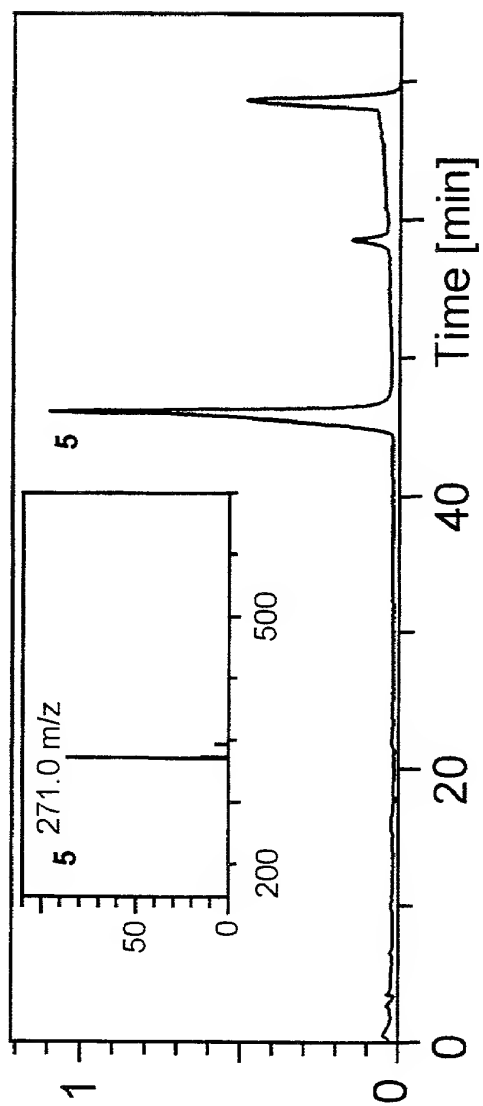


Fig. 7D

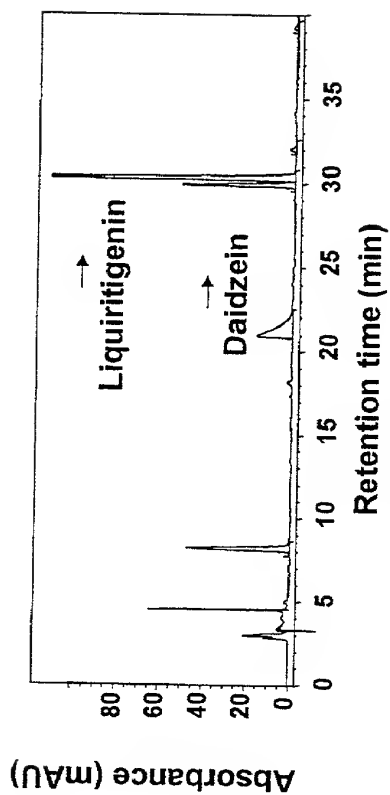


Fig. 8A

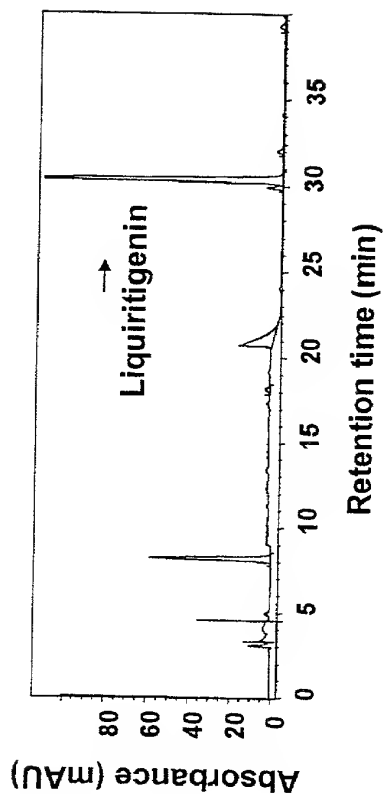


Fig. 8B

14/15

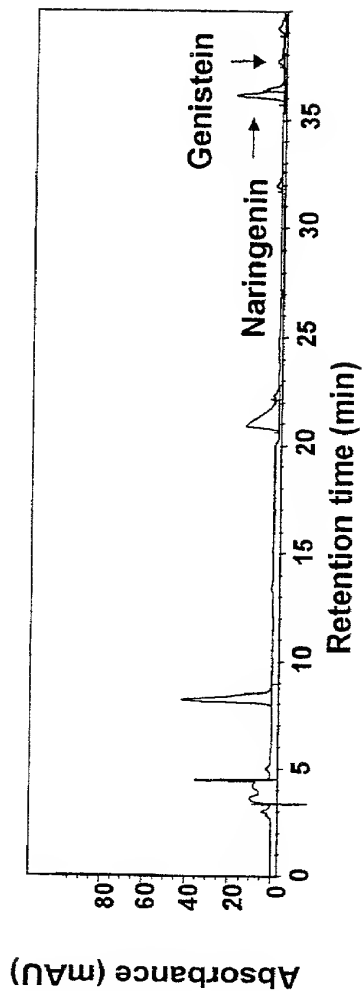


Fig. 8C

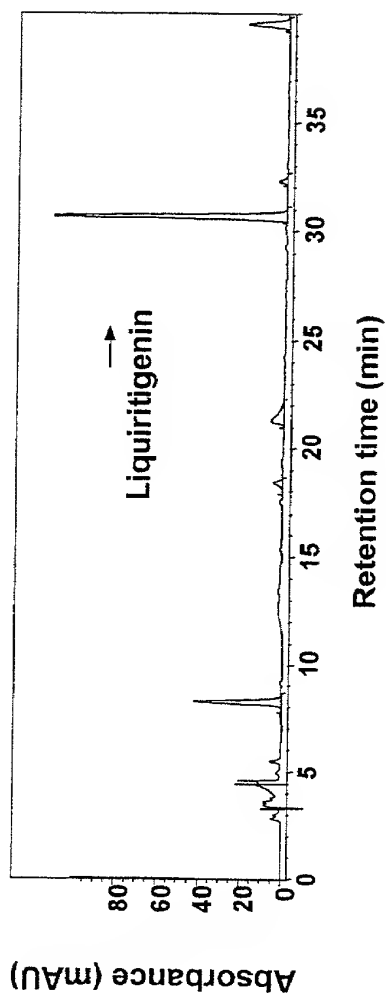


Fig. 8D

Fig. 9A

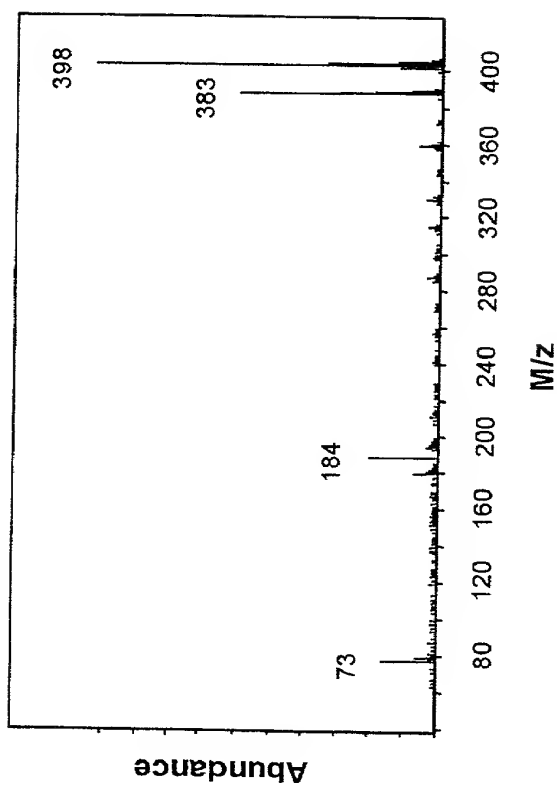
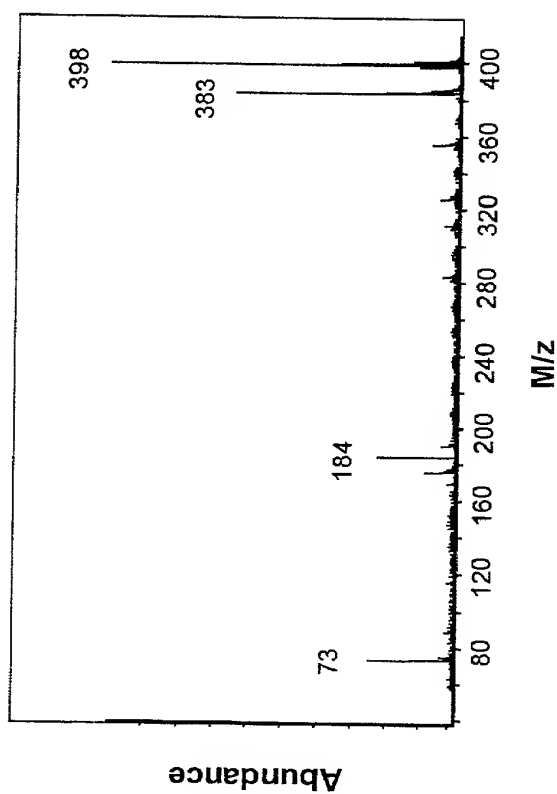


Fig. 9B





**DECLARATION AND POWER OF ATTORNEY**

As a below-named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name, that I believe that I am the original, first and sole inventor (if only one name is listed below) or I believe that we are the original, first and joint inventors (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention, design or discovery entitled **GENETIC MANIPULATION OF ISOFLAVONOIDS**, the specification of which (check one)

- ( ) is attached hereto; or  
 (X) was filed on **March 8, 2000**, as PCT International Application Number  
**PCT/US00/05915**  
 (X) and was amended on or about May 1, 2001.

that we have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above; that we do not know and do not believe that said invention, design or discovery was ever known or used in the United States of America before my invention or discovery thereof, or patented or described in any printed publication in any country before our invention or discovery thereof, or more than one year prior to this application, or in public use or on sale in the United States of America more than one year prior to this application; that said invention, design or discovery has not been patented or made the subject of an inventor's certificate issued prior to the date of this application in any country foreign to the United States of America on an application filed by us or our legal representatives or assigns; and that we acknowledge the duty to disclose information of which we are aware which is material to the examiner of this application in accordance with 37 C.F.R. § 1.56(a).

We hereby claim foreign priority benefits under 35 U.S.C. § 119 of any foreign application(s) for patent or inventor's certificate listed below and have also identified below any foreign application(s) for patent or inventor's certificate having a filing date before that of the application on which priority is claimed:

NUMBER	COUNTRY	DATE FILED	PRIORITY CLAIMED
N/A			

We hereby claim the benefit under Title 35, United States Code, § 119(e) of any United States provisional application(s) listed below.

APPLICATION SERIAL NO.	DATE FILED
U.S. 60/123,267	March 8, 1999

We hereby claim the benefit under 35 U.S.C. § 120 of any United States Application(s) listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States Application(s) in the manner provided by the first paragraph of 35 U.S.C. § 112, I acknowledge the duty to disclose material information as defined in 37 C.F.R. § 1.56(a) which occurred between the filing date of the prior application(s) and the national or PCT international filing date of this application:

APPLICATION SERIAL NO.	DATE FILED	STATUS
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N/A		
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I hereby appoint:

3 Eugenia S. Hansen

Reg. No. 31,966

Karen L. Knezek

Reg. No. 39,253

Rod A. Cooper

Reg. No. 42,436

all of the firm of Sidley Austin Brown & Wood, our attorneys with full power of substitution and revocation, to prosecute this application and to transact all business in the United States Patent and Trademark Office connected therewith.

Send correspondence to:

Direct telephone calls to:

Eugenia S. Hansen

Eugenia S. Hansen

Sidley Austin Brown & Wood

at (214) 981-3315

717 N. Harwood, Suite 3400

Atty. Docket No. **11137/05006**

Dallas, TX 75201

We hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under § 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Full name of sole or joint inventor: Richard A. Dixon

Inventor's signature: MA Dixon

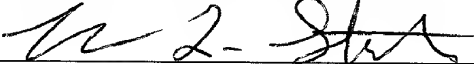
Date: September 10, 2001

Residence (City, County, State): Ardmore, Carter County, Oklahoma OK

Citizenship: **Great Britian**

Post Office Address: **206 Woods Lane, Ardmore, Oklahoma 73401**

Full name of sole or joint inventor: <sup>20</sup>Christopher L. Steele

Inventor's signature: 

Date: 9/6/01

Residence (City, County, State): Manlius, Onondaga County, New York <sup>NY</sup>

Citizenship: **United States of America**

Post Office Address: **7660 Farmington Road, Manlius, New York 13104**

11137/05006

## SEQUENCE LISTING

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ctt tcc tcc taaaatatct tgagaggatg aatcaccaac atatagcctc 1697  
 Leu Ser Ser  
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tctttggtac tacaaaatta tgatgtaatt ttcttatttt ttctgtcaca aaggaagtgt 1757

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Val Val Ala Ser Thr Pro Asp Leu Phe Lys Leu Phe Leu Gln Thr His  
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Glu Ala Thr Ser Phe Asn Thr Arg Phe Gln Thr Ser Ala Ile Ser Arg  
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Leu Thr Tyr Asp Asn Ser Val Ala Met Val Pro Phe Ala Pro Tyr Trp  
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 Lys Phe Ile Arg Lys Leu Ile Met Asn Asp Leu Leu Asn Ala Thr Thr  
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 Glu Glu Leu Leu Lys Trp Thr Asn Ser Thr Ile Ser Thr Met Met Leu  
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 Gly Glu Ala Glu Glu Val Arg Asp Ile Ala Arg Asp Val Leu Lys Ile  
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 Phe Gly Glu Tyr Ser Val Thr Asn Phe Ile Trp Pro Leu Asn Lys Phe  
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 Asp Pro Ile Ile Glu Lys Val Ile Lys Lys Arg Gln Glu Ile Val Asn  
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 Lys Arg Lys Asn Gly Glu Ile Val Glu Gly Glu Gln Asn Val Val Phe  
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 Gly Thr Asp Ser Thr Ala Val Ser Thr Glu Trp Thr Leu Ser Glu Leu  
 305 310 315 320  
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 325 330 335  
 Val Val Gly Lys Asp Arg Leu Val Asp Glu Ser Asp Val Gln Asn Leu  
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 Pro Tyr Ile Lys Ala Ile Val Lys Glu Ala Phe Arg Leu His Pro Pro  
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 Gly Arg Asp Pro Lys Tyr Trp Val Lys Pro Leu Glu Phe Arg Pro Glu  
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11137/05006

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420 425 430

Arg Gly Gln His Phe Thr Leu Leu Pro Phe Gly Ser Gly Arg Arg Met  
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Cys Pro Gly Val Asn Leu Ala Thr Ala Gly Met Ala Thr Met Ile Ala  
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Ser Ile Ile Gln Cys Phe Asp Leu Gln Val Pro Gly Gln His Gly Glu  
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Ala Gly Val Ala Asp Lys Leu Leu Ser Ser  
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## SEQUENCE LISTING

<110> THE SAMUEL ROBERTS NOBLE FOUNDATION, INC.  
 STEELE, Christopher L.  
 DIXON, Richard A.

<120> GENETIC MANIPULATION OF ISOFLAVONOIDS

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Phe Ser Leu Tyr Phe Gly Ser Met Pro Thr Val Val Ala Ser Thr Pro
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Val Ile Lys Lys Arg Arg Glu Ile Val Arg Arg Arg Lys Asn Gly Glu	
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 Pro Tyr Ile Arg Ala Ile Val Lys Glu Thr Phe Arg Met His Pro Pro  
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 Asn Val Ser Asp Phe Ile Trp Leu Phe Lys Lys Met Asp Leu Gln Gly  
 210 215 220  
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 245 250 255  
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 Val Val Gly Lys Asp Arg Leu Val Glu Glu Ser Asp Cys Pro Asn Leu  
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Tyr Val Ile Pro Glu Asp Ser Leu Leu Phe Val Asn Val Trp Ser Ile  
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Arg Phe Leu Lys Leu Glu Gly Asp Ser Ser Gly Val Val Asp Val Arg  
420 425 430

Gly Ser His Phe Gln Leu Leu Pro Phe Gly Ser Gly Arg Arg Met Cys  
435 440 445

Pro Gly Val Ser Leu Ala Met Gln Glu Val Pro Ala Leu Leu Gly Ala  
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Ile Ile Gln Cys Phe Asp Phe His Val Val Gly Pro Lys Gly Glu Ile  
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Leu Lys Gly Asp Asp Ile Val Ile Asn Val Asp Glu Arg Pro Gly Leu  
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Thr Leu Leu Leu Ile Ala Leu Phe Leu His Leu Arg Pro Thr Pro Thr  
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gca aaa tca aag gct ctt cgc cac ctt cca aat cca cca agc cct aaa 208  
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Pro Arg Leu Pro Phe Ile Gly His Leu His Leu Leu Asp Asn Pro Leu  
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Arg Phe Gln Thr Ser Ala Ile Ser Arg Leu Thr Tyr Asp Asn Ser Val	
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Ala Met Val Pro Phe Ala Pro Tyr Trp Lys Phe Ile Arg Lys Leu Ile	
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Asn Leu Met Cys Val Pro Leu Ala Arg Ala Gly Val Ala Asp Lys Leu	
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Leu Ser Ser	
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Pro Asn Pro Pro Ser Pro Lys Pro Arg Leu Pro Phe Ile Gly His Leu
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His Leu Leu Asp Asn Pro Leu Leu His His Thr Leu Ile Lys Leu Gly
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 65             70             75             80

Val Val Ala Ser Thr Pro Asp Leu Phe Lys Leu Phe Leu Gln Thr His
      85             90             95

Glu Ala Thr Ser Phe Asn Thr Arg Phe Gln Thr Ser Ala Ile Ser Arg
      100            105            110

Leu Thr Tyr Asp Asn Ser Val Ala Met Val Pro Phe Ala Pro Tyr Trp
      115            120            125

Lys Phe Ile Arg Lys Leu Ile Met Asn Asp Leu Leu Asn Ala Thr Thr
      130            135            140

Val Asn Lys Leu Arg Pro Leu Arg Ser Arg Glu Ile Leu Lys Val Leu
      145            150            155            160

Lys Val Met Ala Asn Ser Ala Glu Thr Gln Gln Pro Leu Asp Val Thr
      165            170            175

Glu Glu Leu Leu Lys Trp Thr Asn Ser Thr Ile Ser Thr Met Met Leu
      180            185            190

Gly Glu Ala Glu Glu Val Arg Asp Ile Ala Arg Asp Val Leu Lys Ile
      195            200            205

Phe Gly Glu Tyr Ser Val Thr Asn Phe Ile Trp Pro Leu Asn Lys Phe
      210            215            220

Lys Phe Gly Asn Tyr Asp Lys Arg Thr Glu Glu Ile Phe Asn Lys Tyr
      225            230            235            240

Asp Pro Ile Ile Glu Lys Val Ile Lys Lys Arg Gln Glu Ile Val Asn
      245            250            255

Lys Arg Lys Asn Gly Glu Ile Val Glu Gly Glu Gln Asn Val Val Phe
      260            265            270

Leu Asp Thr Leu Leu Glu Phe Ala Gln Asp Glu Thr Met Glu Ile Lys
      275            280            285

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Ile Thr Lys Glu Gln Ile Lys Gly Leu Val Val Asp Phe Phe Ser Ala  
 290 295 300  
 Gly Thr Asp Ser Thr Ala Val Ser Thr Glu Trp Thr Leu Ser Glu Leu  
 305 310 315 320  
 Ile Asn Asn Pro Arg Val Leu Lys Lys Ala Arg Glu Glu Ile Asp Ser  
 325 330 335  
 Val Val Gly Lys Asp Arg Leu Val Asp Glu Ser Asp Val Gln Asn Leu  
 340 345 350  
 Pro Tyr Ile Lys Ala Ile Val Lys Glu Ala Phe Arg Leu His Pro Pro  
 355 360 365  
 Leu Pro Val Val Lys Arg Lys Cys Thr Gln Glu Cys Glu Ile Asp Gly  
 370 375 380  
 Tyr Val Val Pro Glu Gly Ala Leu Ile Leu Phe Asn Val Trp Ala Val  
 385 390 395 400  
 Gly Arg Asp Pro Lys Tyr Trp Val Lys Pro Leu Glu Phe Arg Pro Glu  
 405 410 415  
 Arg Phe Ile Glu Asn Val Gly Glu Gly Glu Ala Ala Ser Ile Asp Leu  
 420 425 430  
 Arg Gly Gln His Phe Thr Leu Leu Pro Phe Gly Ser Gly Arg Arg Met  
 435 440 445  
 Cys Pro Gly Val Asn Leu Ala Thr Ala Gly Met Ala Thr Met Ile Ala  
 450 455 460  
 Ser Ile Ile Gln Cys Phe Asp Leu Gln Val Pro Gly Gln His Gly Glu  
 465 470 475 480  
 Ile Leu Asn Gly Asp Tyr Ala Lys Val Ser Met Glu Glu Arg Pro Gly  
 485 490 495  
 Leu Thr Val Pro Arg Ala His Asn Leu Met Cys Val Pro Leu Ala Arg  
 500 505 510  
 Ala Gly Val Ala Asp Lys Leu Leu Ser Ser  
 515 520